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TITLE OF THESIS..... The purification of IgM from normal human serum .....

.....

UNIVERSITY.... of ALBERTA .....

DEGREE FOR WHICH THESIS WAS PRESENTED.... M.Sc. .....

YEAR THIS DEGREE GRANTED.... 1974 .....

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DATED.... 4 october 1974

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THE UNIVERSITY OF ALBERTA

THE PURIFICATION OF IgM FROM NORMAL HUMAN SERUM

by

FABIENNE BECKERS

A THESIS

SUBMITTED TO

THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT

OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF BACTERIOLOGY

EDMONTON, ALBERTA

FALL, 1974

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled The Purification of IgM from Normal Human Serum submitted by Fabienne Beckers in partial fulfilment of the requirements for the degree of Master of Science.

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Supervisor

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Date..23.Sept.....1974.....

ABSTRACT

An attempt was made to develop a convenient large scale procedure for the isolation of IgM from normal human serum.

Three procedures were tested. Precipitation with dextran sulfate, ammonium sulfate and polyethylene glycol-6000, followed by chromatography on DEAE-cellulose columns was used in each procedure. Further purification of IgM was obtained either by gel filtration, or by further precipitation with 4% PEG-6000, or by a combination of precipitation with 5% PEG and gel filtration.

The recovery of IgM in the final preparations varied from 18.1% to 23.5% to 23.3% for the three procedures used. Calculations indicated that the IgM content in the final preparations was 82% (A), 75% (B) and 95.2% (C) of the total protein content. However, the ultracentrifugation and immunoelectrophoretic patterns showed no major differences between preparations B and C.

In conclusion, a relatively simple large scale procedure for purification of IgM was developed, resulting in a preparation with 20-25% yield and 95% purity.

ACKNOWLEDGEMENTS

We very gratefully acknowledge Doctor C.R. Shobe for his supervision and kind support, without which this thesis would never have been possible.

We also wish to express our gratitude to Doctor Jackson, Chairman of the Department of Bacteriology.

Many thanks are also due to Doctor K.W. Walker, Director, Division of Biochemistry, for his invaluable help with the immunoelectrophoresis and quantitation of immunoglobulins and α_2-macroglobulin.

The ultracentrifugation of our final samples was made possible through the kind collaboration of Doctor J. Kinear.

Normal human serum was generously supplied by the Provincial Laboratory of Public Health.

Finally, we thank Ortella White for her patience during the typing of this manuscript.

TABLE OF CONTENTS

Introduction.....1

I. General aspects.....1

II. Purification of IgM.....3

A. Precipitation techniques.....4

1. Precipitation by removal of electrolytes.....4

2. Fractional precipitation by neutral salts.....6

3. Fractionation by water soluble non-ionizable linear polymers.....8

B. Chromatography.....10

1. Gel filtration.....10

2. Ion exchange chromatography.....14

3. Fractionation of proteins with insolubilized concanavalin A.....16

Materials and Methods.....19

A. Materials.....19

B. Methods.....20

1. Analytical methods.....20

i. Protein measurements.....20

ii. Qualitative immunodiffusion assays.....20

iii. Quantitative determination of Ig's and α_2-macroglobulin.....21

iv. Immunoelectrophoresis.....21

v. Ultracentrifugation.....22

2. Exchange of buffers.....22

3. Concentration of protein solutions.....23
C. Experimental procedures.....23
1. Purification sequence A.....23
Part I. Precipitation methods.....23
i. Precipitation with dextran sulfate.....23
ii. Precipitation with ammonium sulfate.....24
iii. Precipitation with polyethylene glycol-6000 (PEG-6000).....24
Part II. Chromatography.....25
i. Anion-exchange chromatography.....25
ii. Gel filtration.....27
2. Purification sequence B.....28
Part I. Precipitation methods.....28
Part II. Anion-exchange chromatography.....29
Part III. Second precipitation with PEG-6000.....29
3. Purification sequence C.....29
Part I.....29
Part II. Gel filtration on Sepharose 4B.....30
Results.....31
Part I. Precipitation methods.....31
Part II. Chromatography and selective precipitation.....34
Procedure A.....34
i. Anion-exchange chromatography.....34
ii. Gel filtration.....38
iii. Analysis of final IgM preparation.....44
Procedure B.....50

i. Anion-exchange chromatography.....50

ii. Precipitation with 4% PEG.....52

iii. Analysis of final IgM Preparation (Procedure B)....53

Procedure C.....54

i. Precipitation with 5% PEG and gel filtration on Sepharose 4B.....54

ii. Analysis of final IgM preparation.....54

Discussion.....62

Bibliography.....68

Appendix 76

Bibliography to appendix.....80

LIST OF TABLES

Table 1. Procedure A..... 32

Table 2. Second part of procedures B and C..... 51

LIST OF FIGURES

Fig. 1. Chromatography on DEAE-cellulose..... 36

Fig. 2. Chromatography on Sephadex G-200..... 40

Fig. 3. Chromatography on Sepharose 4B..... 43

Fig. 4. Immunoelectrophoretic pattern of IgM preparation from procedures A and B..... 46

Fig. 5. Ultracentrifugation of IgM preparation from procedure A.... 48

Fig. 6. Immunoelectrophoretic pattern of IgM-containing solution obtained after precipitation with 5% PEG..... 56

Fig. 7. Immunoelectrophoretic pattern of IgM preparation from procedure C..... 59

Fig. 8. Ultracentrifugation of the IgM preparations from procedures B and C..... 61

INTRODUCTION

I. *General aspects*

The principal immunoglobulins in normal human serum are IgG, IgA and IgM at concentrations of 600 - 1800 mg (70 - 210 I.U.), 60 - 450 mg (38 - 272 I.U.) and 60 - 220 mg (68 - 250 I.U.) per 100 ml respectively (3). Lesser amounts of IgD (3 mg/100 ml) (77) and IgE (0.1 mg/100 ml) (41) are also found and together the immunoglobulins constitute about 20% of the total plasma proteins (3).

The feature which distinguishes IgM from the other immunoglobulins is its high molecular weight. On ultracentrifugation, IgM preparations are usually heterogeneous, with the major component having a sedimentation coefficient of 19 S corresponding to a molecular weight (M.W.) of 900,000 - 1,000,000 (74), but components with higher sedimentation rates are usually present as well (23, 79). These heavy components are thought to arise from aggregation or polymerization of the 19 S component (58).

A similar heterogeneity is encountered in IgA preparations. The main component of purified IgA preparations has the same molecular weight and sedimentation coefficient as IgG (160,000 and 6.6 - 7 S respectively) (74), but minor amounts of 10.5 S, 13 S and 15 S species of IgA having molecular weights up to 500,000 are also seen on ultracentrifugation (15, 35).

The isoelectric points of IgM molecules vary between pH 5.5 and pH 7.4, so that on immunoelectrophoresis at pH 8.6, IgM is seen throughout the γ-globulin region, extending into the β-globulin region

as well. Similar electrophoretic mobilities are observed for IgA (pI: 4.8 - 6.5) and IgG (pI: 6.2 - 8.5). Most of the IgA and IgG is found in the slow β region and mid-γ region, respectively, while most of the IgM is found in the fast γ region (56, 74).

Structurally, IgM is considered to be a pentamer of subunits linked together by disulfide bonds (59). Each subunit, consisting of two light chains and two heavy chains, resembles the IgG molecule, but the subunits of IgM have been found to have a higher molecular weight (59). The carbohydrate content of IgM (10 - 12%) is also much higher than that of IgG (2 - 3%) (15). Most of the carbohydrate is associated with the heavy chains; IgG contains only one glycopeptide per heavy chain (γ chain), while IgM contains 4 to 6 glycopeptides per heavy chain (μ chain) (12, 82).

Biologically, IgM is characterized by its early appearance in the immune response, providing a potential early diagnostic tool in the detection of certain diseases (e.g. Rubella (5)).

Human immunoglobulin M is difficult to isolate from normal human serum because of its heterogeneous character and because so little is present. Therefore, pathological sera (Waldenström sera) are often used as a source of IgM. The large amounts of paraprotein present in these sera favor its isolation and moreover a decrease in the concentrations of other immunoglobulins, both absolute and relative, is often found as well, so that the amounts of other globulins from which IgM must be separated are reduced (21, 56).

Waldenström proteins are considered to arise from a massive production of one of the many different IgM species present in normal

serum (57). In contrast with IgM purified from normal serum, Waldenström proteins are characteristically homogeneous, except for their sedimentation coefficients where higher values than 19 S are also found, indicating that aggregation of Waldenström proteins also occurs (19). On electrophoresis they are found as a narrow peak in the γ or β region, rather than extending as a broad zone over the whole γ-β region (13, 72).

The antigenic specificity associated with pathological immunoglobulins seems to be directed against specific antigens, in contrast with the wide range of antigenic specificities found in normal immunoglobulin M (57).

Although studies on pathological IgM provide useful information on the structure and functional characteristics of IgM, ultimate answers must come from studies on pure normal IgM, since extrapolation of data arising from studies on pathological or atypical macroglobulins always involves some risk of misinterpretation (58).

II. *Purification of IgM*

IgM is commonly isolated by following an initial selective precipitation with other fractionation methods based primarily on molecular size and/or change.

Precipitation techniques, although lacking the specificity necessary to give clear-cut fractionations, are used primarily as a preparative step to reduce the sample volume and the amount of proteins other than IgM which must be processed before other more specific fractionation methods are applied. Several precipitation techniques can be used, the two most "classical" methods being precipitation by

removal of electrolytes and precipitation by neutral salts. An additional technique which has been introduced recently and which seems to give a more selective separation of proteins involves precipitation with water soluble, non-ionizable polymers.

More specific fractionation methods which are based on charge include zone electrophoresis and ion-exchange chromatography. Separation on the basis of size can be affected by either gel filtration or ultracentrifugation. However, ultracentrifugation and electrophoresis are usually more useful for microscale preparation and can represent rather complicated techniques when applied on a macroscale as preparative procedures. An additional chromatographic technique, involving chromatography on insolubilized concanavalin A columns, which separates proteins on the basis of their carbohydrate content, has also been developed in recent years.

A. *Precipitation techniques*

1. *Precipitation by removal of electrolytes*

Early classifications, based partly on solubility properties, divided serum proteins into albumin and globulin fractions. The globulin fraction could be subdivided following dialysis against low ionic strength buffers or distilled water since this procedure gave rise to two subfractions, one containing precipitated globulins referred to as euglobulins and another containing soluble globulins, called pseudoglobulins (16).

The main components of the euglobulin precipitate were found to be β-lipoproteins, ceruloplasmin, macroglobulins (especially IgM) and some IgG and haptoglobin (81). However, when serum was dialized against

low ionic strength buffers instead of distilled water, the composition of the euglobulin precipitate was found to depend on the acidity and the ionic strength of the buffers employed (78). The reasons for this are as follows:

(i) a particular protein in solution is least soluble at or near its isoelectric point (pI), so that in a solution containing a mixture of proteins, those with pI's closest to the pH of the solvent will be preferentially precipitated (16).

(ii) when euglobulins are suspended in buffers of relatively high ionic strength, they dissolve readily. When, at a given pH, the ionic strength of the solution is gradually decreased, each species of protein will begin to precipitate when the ionic strength drops below that level required to maintain that particular protein in solution (16). For instance, when normal human serum is dialyzed against 0.002 M phosphate buffer, pH 6.0, the precipitate consisting mainly of β-lipoproteins and macroglobulins (including IgM) can be obtained (7).

Euglobulin precipitation has been found to be particularly useful in the purification of many pathological species of immunoglobulin M. Waldenström macroglobulins usually show a pronounced euglobulin character and precipitate readily when dialyzed against distilled water or dilute buffers (73). However, Waldenström IgM's with no euglobulin character have also been observed and other preparative techniques are required for their purification (13, 29, 42, 64, 87). Euglobulin precipitation has also been applied to the isolation of normal human IgM (7, 75, 90), but it has been reported that some of

the normal IgM molecules behaved as though they were pseudoglobulins so that complete precipitation of the IgM fraction could not be achieved (90).

2. *Fractional precipitation by neutral salts*

Dilute solutions of neutral salts often increase the solubility of proteins. This phenomenon, called "salting-in", is a consequence of the interaction of the ions from the salt with the ionizable groups of the protein (65). At higher salt concentrations, however, salt ions attract the more polarizable water molecules of the medium, thus interfering with interactions between water molecules and charged polar groups on the proteins with the result that precipitation ("salting-out") of the proteins will occur (65).

Several neutral salts (phosphates, sulfates, citrates and chlorides) have been used for protein fractionation, but ammonium sulfate and sodium sulfate are most commonly used at the present time. Although ammonium sulfate is theoretically less effective for this purpose than sodium sulfate, its higher water solubility, which varies less with temperature than does the solubility of sodium sulfate, makes it a more readily controllable and therefore more useful precipitating agent (16).

Ammonium sulfate has been used to separate plasma proteins into various fractions, including fibrinogen which is precipitable at 20 - 25% saturation with ammonium sulfate, euglobulins, which precipitate at 28 - 33% saturation, and pseudoglobulins, which precipitate between 33 and 50% saturation with the salt (16). Albumin and some α-globulins

are found in the supernatant after precipitation at 50% saturation (80). It is worthwhile mentioning that the "euglobulin" and "pseudoglobulin" fractions obtained by ammonium sulfate precipitation differ in composition from those obtained by dialysis of serum against distilled water. The "euglobulin" fraction obtained by ammonium sulfate precipitation contains many globulins which are soluble in distilled water, while about 20% of the "pseudoglobulin" fraction consists of distilled water insoluble globulins (16).

Except for a single, unique γ-globulin preparation, attempts to use ammonium sulfate to fractionate the globulins into specific high purity preparations have been unsuccessful (81). Nevertheless, precipitation with ammonium sulfate has been used often as a preliminary step in the purification of normal IgM because of the ease and utility with which the technique can be applied. Although also applicable to pathological sera, it usually replaces the euglobulin precipitation technique only when the pathological IgM is found to have no euglobulin character (33, 42, 64).

The concentration of ammonium sulfate required to precipitate IgM from a protein solution seems to be variable. This is especially true for the macroglobulinemic IgM's which have been found to differ markedly in their solubility properties (33, 54, 55, 73). Different normal sera may also require different concentrations of ammonium sulfate for complete precipitation of IgM, since at pH 7.0, concentrations varying from 1.2 M (~28% saturation) to 2.05 M (~50% saturation) ammonium sulfate have been found to be necessary by different authors (63, 79, 80).

3. *Fractionation by water soluble non-ionizable linear polymers*

Polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), nonylphenol-ethoxylate (NPE), polyethylene glycol (PEG) and dextrans have been shown to act as protein precipitants with fractionating properties. Dextrans and particularly PEG appear to be most suitable for this purpose since they lack the high intrinsic viscosity and the denaturing properties of the other polymeric precipitants. Moreover, PEG with a molecular weight of 6000 (PEG-6000) may be the polymer of choice, since it lacks the viscosity of higher molecular weight PEG polymers and is a more selective precipitant than the lower molecular weight PEG derivatives (67).

The mechanism underlying the precipitating properties of these polymers is considered by most investigators to be different from neutral salt precipitation mechanisms (43, 48, 67, 68). The observation that low concentrations of high molecular weight polymers were as effective as high concentrations of low molecular weight polymers in precipitating proteins, lead Polson *et al.* (67) to conclude that these effects could not be explained by a dehydration model. In fact, if the mechanism was based on dehydration, equal weights of low molecular weight polymers should have been more effective as a result of the higher osmotic pressure obtained (67). Laurent (48) suggested that perhaps the polymer might sterically exclude proteins from interacting with the solvent. This "exclusion" theory has been supported by the observation that an increase in the molecular weight of the polymer does not result in an increased efficiency of precipitation once a certain optimum molecular weight has been reached (43, 67). For example, PEG of molecular weight 6000 (PEG-6000) is just as effective a precipitant as PEG of molecular weight 20,000.

According to Laurent (48), the concentration of a polymer required to precipitate a protein will depend only on the size of the protein. Although it has been found that large proteins generally are precipitated at lower polymer concentrations (40, 43), fractionation of plasma proteins does not strictly follow this rule. For example, with PEG-6000 at pH 8.0, IgG (MW: 160,000) precipitates at the same PEG concentration as α_2-macroglobulin (MW: 820,000) (8). It may be concluded, therefore, that as in other fractionation methods the charge on the protein molecule plays some role in determining its solubility characteristics. In other words, the concentration of polymer required to precipitate a particular protein is still dependent on the pH of the solution and the pI of the protein, even though the mechanism for inducing precipitation may be different from that involved in "salting-out" techniques. Polson *et al.* (67) have reported that at pH 7.0, serum proteins were precipitated by PEG-6000 in order of increasing electrophoretic mobility but that at pH 4.6 the order of precipitation was reversed. As well, Polson *et al.* (67) and others (40, 43, 67) have suggested that the efficiency and resolution of the PEG-6000 fractionation procedure was enhanced at elevated temperatures and lower initial protein concentration. Polson *et al.* (67) also suggested that variations in ionic strength had little influence on protein fractionation by this method, but Chun *et al.* (11) have disagreed with that conclusion.

Since the introduction of PEG-600 as a protein fractionating agent (67), it has become widely used for this purpose. Gamma globulins (67), fibrinogen (67) and albumin (43, 67) have been isolated by PEG precipitation in combination with ammonium sulfate precipitation. In

general, the yields and purity have been superior to those resulting from other fractionation methods. Chun *et al.* (11) attempted to isolate IgG from commercially prepared immunoglobulins and from whole human serum by sequential precipitation at different PEG concentrations. The end product was reported to be quite homogeneous but some contamination with IgM and IgA could not be eliminated. Chesebro and Svehag (8) found that IgM could be precipitated completely from serum by 7% PEG-6000 at pH 8.0. At this concentration, however, other proteins including α_2- and β-lipoproteins and some of the α_2-macroglobulin, IgA and IgG were also precipitated. Nevertheless, this approach provides a convenient and useful way of concentrating IgM prior to the application of subsequent, more selective fractionation procedures (8, 9).

B. *Chromatography*

1. *Gel filtration*

Chromatography on gels of cross-linked dextran, agarose or acrylamide can be used to separate substances with different molecular dimensions. The separation mechanism can be explained as follows:

(i) The separating gel column has a mobile phase and a stationary phase, the latter consisting of gel beads formed through cross-linking of a monomer. When suspended in solvent these beads swell and solvent enters the interior of the beads. The volume of solvent within these beads is referred to as the inner volume (Vi). The mobile phase is formed by the solvent outside of the beads and is called the void volume (Vo) (27, 88).

(ii) When a solution is applied to a column of separating gel, the solute molecules distribute freely in the void volume. Whether or not the solute molecules penetrate into the beads and to what extent they do so depends on the size of the pores leading to the interior of the gel beads in relation to the size of the solute molecules (88). Highly cross-linked gels have small pores and allow only small molecules to penetrate, while the interiors of less extensively cross-linked gels are accessible to larger molecules (70).

(iii) For a particular type of gel, molecules too large to diffuse into the interior of the beads pass unhindered through the column and have an elution volume (Ve) equal to the void volume (49). Ve is defined as the volume of liquid passing through the gel from the moment of application of the substance to the moment of its emergence from the column in maximum concentration (86). For small molecules entering all regions of the separating gel displacement of the entire volume of the gel bead (Vt) is required and then Ve is therefore close to Vo + Vi. Between these two extremes (Vo and Vt) lies the fractionation range of the gel. Molecules of intermediate size will have access to the gel phase in a proportion which is inversely related to their molecular size. Each type of molecule is characterized by its distribution coefficient (Kd) which is that portion of the Vi to which the molecules in question have access (27, 49). The volume of the solvent occupied by a substance is thus Kd Vi + Vo. Since the distribution volume of a substance is equal to its elution volume,

it can be said that its Ve = Vo + Kd Vi (27, 49).

Porath and Flodin (69) used highly cross-linked dextran gels for desalting of proteins. Subsequently, less extensively cross-linked dextran gels (Sephadex) have been developed which allowed fractionation of proteins of higher molecular weight.

Sephadex G-200 with the lowest degree of cross-linkage in this series and with a fractionation range of 5,000 to 800,000 (26) appears appropriate for fractionation of plasma or serum proteins. Flodin and Killander (28) showed that when serum is chromatographed on Sephadex G-200, the proteins are eluted in three peaks. The first peak contained macroglobulins (α_2-macroglobulin, α- and β-lipoproteins and IgM), the second peak contained mainly 7 S γ-globulins along with α- and β-globulins and the third peak consisted mainly of albumin (28). Other investigators found similar elution patterns (25, 31, 47, 93), although some have reported that haptoglobin was also eluted in the first peak (25, 93). Fibrinogen was found in the macroglobulin peak when plasma was fractionated on Sephadex G-200 (31, 47).

Studies on the elution behavior of antibodies revealed that 19 S γ-globulins (IgM) and 7 S γ-globulins (IgG) were eluted in the first and second peaks respectively, thus providing a useful means of separating IgM and IgG (39, 45, 46, 76). Most of the IgA, which has a molecular weight intermediate between that of IgM and IgG, was eluted in the trough between the first and the second peak. It was found partly in the descending portion of the first peak and partly in the ascending portion of the second peak, implying that complete separation of IgA from IgM or IgG was impossible by chromatography on Sephadex G-200

(24, 25, 39, 45, 46, 76).

Cross-linked polyacrylamide gel (Biogel P series) have fractionation ranges similar to those of the Sephadex type which have a corresponding water regain (26) and they also show a similar distribution of serum proteins on chromatography (36, 38). Although most of the serum proteins fall within the fractionation ranges of polyacrylamide and dextran gels, IgM and other macroglobulins are eluted with the void volume and cannot be separated.

Agarose gels, first introduced in beaded form by Hjertén (37) and Bengtsson and Philipson (4) are now available under the designations Sepharose and Biogel A. They complement the use of dextran or polyacrylamide gels for serum protein fractionation since the molecular weights of serum macroglobulins fall within the fractionation ranges of the agarose gels (50,000 up to several millions) (26). Killander *et al.* (47) subjected the macroglobulin peak (first peak) of Sephadex G-200 fractionated normal human plasma to chromatography on an agarose gel equivalent to those currently designated as Sepharose 4B or Biogel A 1.5. The first half of the IgM-containing fractions were free of α_2-macroglobulin and α_1-lipoproteins (high density lipoproteins) (47). When longer columns or recycling chromatography (71) was used, IgM was almost completely separated from α_2-macroglobulin and no contamination by fibrinogen (47), haptoglobin (10) or IgA (10) was detected. However, α_2- and β-lipoproteins (low density lipoproteins) were still found throughout the whole IgM peak (47) and had to be removed by other methods (10, 47).

2. *Ion exchange chromatography*

Chromatography on ion-exchange cellulose was introduced by Peterson and Sober (66) in 1956. Ion-exchangers prepared by substitution of the hydroxyl groups of cellulose with various derivatives form the basis for this approach to protein purification. In particular, the cation-exchanger carboxymethyl cellulose (CM-cellulose) and the anion-exchanger diethylaminoethyl cellulose (DEAE-cellulose) have been applied widely in the fractionation of serum proteins (17, 66, 83, 84, 89). A greater emphasis has been placed on the use of DEAE-cellulose since it has a relatively high capacity for adsorbing proteins and larger amounts of serum can be fractionated on columns which are of a convenient size (22).

The binding of proteins to DEAE-cellulose anion-exchangers depends mainly on electrostatic interactions between the protein polyelectrolyte and the ionized absorbent. Adsorption of proteins to DEAE-cellulose is enhanced at low salt concentrations and alkaline pH, and desorption is favored by increasing the salt concentration and/or decreasing the pH. Decreasing the pH results in reduction of the number of negative charges on the proteins, while increasing the salt concentration promotes dissociation of the electrostatic linkages between the proteins and the adsorbent (84). Elution of adsorbed proteins can be achieved, therefore, by appropriate step-wise changes in the composition of the eluting buffer, or by buffer gradients. Although step-wise elution is useful in a practical sense as a first step in large scale protein purification procedures, the resolution obtained is inferior to that which is achieved by gradient elution (66).

Analysis of the chromatogram of serum proteins, obtained with concave gradients of increasing molarity and decreasing pH, revealed that serum proteins were generally eluted from DEAE-cellulose in order of increasing electrophoretic mobility (84, 89). The fractions eluted after the main albumin peak are anomalous, however, in that they contain proteins representing every electrophoretic region (17, 84). This may be explained by the fact that the total number of charged groups on a protein molecule determines the extent of the electrostatic interaction between the protein molecule and the adsorbent. A large protein with a low charge density but a greater total number of charges may bind more tightly to an anion-exchanger than a small protein with a high charge density and therefore be eluted later from the column as a consequence (85). For example, it has been found that most of the IgM in human serum is eluted from DEAE-cellulose columns after the main serum albumin peak (18).

Electrophoretic characterization of fractions obtained by DEAE-cellulose column chromatography of human serum, showed that with one exception (IgG peak) none of the protein peaks were electrophoretically homogeneous (17, 66, 89). Consequently, with the exception of IgG, none of the serum proteins can be obtained in a pure state by chromatography on DEAE-cellulose.

Analysis of the elution positions and the compositions of the major immunoglobulin peaks has shown that IgM is eluted from the column last but that it is not completely separated from other immunoglobulins and serum proteins by chromatography on DEAE-cellulose. Most of the IgA-containing peak is eluted before the IgM peak, but over-

lapping of the two peaks occurs. As well, IgM-containing fractions are contaminated with IgG and, in fact, small amounts of IgG are found throughout the whole serum chromatogram (18, 89). In addition to IgA and IgG, other serum proteins, such as albumin (81), α_2-macroglobulin (22), haptoglobin (7), ceruloplasmin (81) and β-lipoproteins (7) are eluted in conjunction with the main IgM peak.

Despite this contamination, however, chromatography on DEAE-cellulose is a useful step in the isolation of IgM since it can be used conveniently to obtain enriched IgM preparations which can then be subjected to further fractionation procedures (7, 20, 30).

3. *Fractionation of proteins with insolubilized concanavalin A*

Concanavalin A (Con. A), a globulin obtained from jack bean (*Canavalia ensiformis*), was first isolated by Sumner (86B) in 1919 and later identified as a hemagglutinin. It forms precipitates with various polysaccharides which have in common branched chains terminating in non-reducing sugars (α-D-glucopyranose, α-D-mannopyranose or β-D-fructofuranose) (1). The Con. A - polysaccharide interaction can be inhibited by low molecular weight carbohydrates such as D-glucose, D-fructose, D-mannose and sucrose (1).

Nakamura *et al.* (61) reported that extracts of jack bean could precipitate several human serum proteins. Later studies with both crude jack bean extracts and Con. A showed that these serum proteins were α_2-macroglobulin, IgM, β-lipoprotein, ceruloplasmin, haptoglobin, IgA, α_1-antitrypsin, transferrin and β_2-glycoprotein (34, 50). Only small amounts of normal IgG (0 - 5%) formed complexes with

Con. A, but in myeloma sera up to 50% of the IgG could be precipitated (50). The myeloma proteins with a β-mobility on immunoelectrophoresis were preferentially precipitated (34). Since these myeloma proteins had a higher carbohydrate content than normal IgG (60), it was suggested that the carbohydrate component of these proteins form precipitating complexes with Con. A (34). This was confirmed later by Goldstein *et al.* (32) who demonstrated that Con. A formed precipitates only with glycoproteins which had the appropriate carbohydrate complexes, as described above.

Recently, unsolubilized Con. A columns, prepared by coupling Con. A to cyanogen bromide activated agarose, have been introduced (14, 51). When mixtures of polysaccharides or glycoproteins were applied to these columns, the immobilized Con. A bound only those substances which had the carbohydrate structure necessary for complex formation. Those substances could then be eluted from the column by inhibitors of the Con. A - polysaccharide interaction (1). Insolubilized Con. A adsorbents have been used in attempts to fractionate serum proteins and Aspberg and Porath (2) found that serum proteins could be divided into "non-adsorbed" and "adsorbed" fractions. The non-adsorbed fraction contained albumin and γ-globulin, while the adsorbed fraction, which was eluted from the adsorbent with 0.1 M methyl-α-D-mannopyranoside, contained α- and β-globulins together with pre-albumin and IgM. The adsorbed fraction contained nearly eight times as much carbohydrate as the unadsorbed peak (2).

Weinstein (91) attempted to separate IgM and IgG on the basis of differences in their carbohydrate contents. He introduced purified

antibodies directed against a defined haptenic determinant into a Con. A - agarose column. The unadsorbed fraction was pure IgG, while the adsorbed fraction, which was eluted with 0.2 M methyl α-D-mannopyranose contained IgM with about 5% of the original IgG as a contaminant. Those experiments demonstrated that a separation of glycoproteins into a group with low carbohydrate content and a group with high carbohydrate content could be readily attained. It was suggested that fractionation of serum proteins in this manner could be improved if the absorbed proteins were eluted with a concentration gradient of the inhibitor rather than by a single step elution technique (2).

Experimental Objectives

The increasing importance of IgM as a diagnostic reagent suggested that a convenient method for large scale IgM purification would be welcomed. It was suggested that by combining some of the classical purification techniques with recent procedures involving selective precipitation with non-ionic polymers such as dextrans and polyethylene glycol, a protocol for efficient large-scale IgM purification might be developed. The following report outlines the experimental approach to the problem of IgM-purification that was followed in this laboratory as well as the results of that experimentation.

Note:A discussion of the clinical relevance of IgM as well as an outline of proposed uses for IgM purified by the procedure described below is included as an Appendix to this thesis.

MATERIALS AND METHODS

A. *Materials*

Pooled human serum was provided by the Provincial Laboratory of Public Health, University of Alberta.

All chemicals were of reagent grade and were obtained from commercial suppliers.

Crystalline bovine serum albumin was supplied by Sigma Chemical Company, St. Louis, Missouri, U.S.A. Polyethylene glycol, M.W. 6000 (PEG-6000) was purchased from J.T. Baker Chemical Co., Phillipsburg, New Jersey. All resins used for gel filtration (Sephadex G-25, Sephadex G-200, Sepharose 4B) came from Pharmacia, Uppsala, Sweden, while DEAE-cellulose (Cellex D) with an exchange capacity of 0.87 meq/gram was purchased from BioRad Lab., Richmond, California.

Human Plasma Fraction II (γ-globulins) were obtained from ICN Ltd., Canada.

The monospecific rabbit and goat antisera against human IgM, IgG, IgA, α_2-macroglobulin and ceruloplasmin, used in the qualitative immunodiffusion assays, were obtained from Pentex, Miles Laboratories, INC Kankakee, Ill. The antisera used in the quantitative single-radial-immunodiffusion assays were: horse anti-human serum from Hyland, Div. Travenol Laboratories, California, U.S.A.; goat monospecific anti-human IgM, anti-human IgA, anti-human IgG sera from Malloy; and rabbit anti-human-α_2-macroglobulin serum from Behring, Behringwerke AG, Marburg-Lahn. The standards used in the quantitative assays were also purchased from Behring.

B. *Methods*

1. *Analytical methods*

i. *Protein measurements*: The protein content of column effluents was monitored by measuring the absorbance of each fraction at 280 nm with a Beckman DB-G spectrophotometer.

Protein concentrations were also determined by the method of Lowry *et al.* (52) using pure crystalline bovine serum albumin as the reference protein. For the determination of protein concentrations in purified IgM preparations, Human Plasma Fraction II was used as the protein standard.

ii. *Qualitative immunodiffusion assays*: Protein-containing fractions obtained at various steps in the IgM purification procedure were assayed qualitatively for IgM, IgA, IgG and, in certain instances, for α_2-macroglobulin and ceruloplasmin by the Ouchterlony double diffusion method (62).

The diffusion medium consisted of 0.9% Noble agar (Difco Lab.) in borate saline (95 parts 0.85% sodium chloride with 5 parts 1.5% sodium borate), pH 8.0, containing 0.2% Buffalo black (Allied Chemical) as a background stain and 0.02% sodium azide
The mixture was autoclaved for 15 min at 120°C, cooled to 60°C and poured into plastic Petri dishes to form a thin flat agar layer. A template, as described below, was placed on top of the solidified agar layer and warm agar was added to form a layer 2.5 mm thick. When the agar had cooled, the template was removed, leaving a regular hexagonal array of wells surrounding a central well in the agar. Two templates

were used, one giving wells with a diameter of 3 mm and an inter-well distance of 7.0 mm, and a second which gave well diameters of 2.5 mm and an inter-well distance of 9.5 mm. The immunodiffusion plates were stored at 4°C until used.

Immunodiffusion assays of column fractions and other samples were normally carried out by placing 10 µl of the sample in one of the peripheral wells and 10 µl of antiserum in the central well. When a particular sample was looked at more closely, the sample was placed in the central well and a variety of antisera were placed in the peripheral wells. The immunodiffusion plates were then placed in glass Petri dishes along with a piece of moistened filter paper and left for 3 days at 18°C. Precipitation lines were detected by visual examination using oblique lighting.

iii. *Quantitative determination of Ig's and* α_2*-macroglobulin*: Quantitative immunodiffusion assays were carried out according to the single-radial-diffusion method of Mancini *et al.* (53). In particular, the concentrations of IgM, IgA and IgG were determined in the starting material (pooled human serum) and in samples obtained at various stages in the purification procedure. As well, purified IgM preparations were assayed quantitatively for α_2-macroglobulin, since contamination with this protein cannot be detected by ultracentrifugation, its sedimentation coefficient being about the same value as that of IgM.

iv. *Immunoelectrophoresis*: The homogeneity of IgM preparations at different stages in the purification sequence was determined by immuno-

electrophoresis. Immunoelectrophoresis was carried out with anti-whole human serum, anti-human IgM, anti-human IgA and anti-human IgG antisera. The purity of the final IgM preparations was judged by comparing the immunoelectrophoretic patterns obtained after reaction with anti-whole human serum against that obtained after reaction with anti-human IgM serum.

An LKB electrophoretic apparatus and power supply were used. Microscope slides were flooded with 1% agar (Difco Lab), dissolved in 0.1 M veronal buffer, pH 8.6. Samples (3 µl) of IgM-containing solutions were placed in the antigen wells and separated by electrophoresis for 60 min at 250 V with a current of 55 - 60 mA. Veronal buffer (0.1 M, pH 8.6) was also used as the conducting buffer. Appropriate antisera were then applied to each trough and the slides placed in a humidified chamber for 20 hours at room temperature. The slides were rinsed with 0.9% saline for 4 hours and stained with 0.6% Amido Schwartz 10B in methanol, acetic acid and distilled water (45:10:45).

v. *Ultracentrifugation:* Ultracentrifugation analysis was carried out in a Beckman Model E ultracentrifuge at 60,000 r.p.m. Purified IgM preparations were resuspended in 0.1 M phosphate buffer, pH 7.0, containing 0.5 M NaCl for this purpose. Photographs taken at 4 min intervals from 8 min to 40 min after reaching full speed were used for determination of the observed sedimentation coefficient. For calculations of S^{w}_{20} a partial specific volume of 0.722 was used (59).

2. *Exchange of buffers*

Between different steps in the procedure it was often

necessary to change from one buffer to another. When large volumes of protein solutions in high ionic strength buffers were involved, exhaustive dialysis at 4°C against large volumes of the appropriate buffer was the method of choice. The dialyzing buffer was changed frequently to ensure optimum results. Protein solutions with volumes of less than 20 ml were passed through a 2.5 x 50 cm Sephadex G-25 column equilibrated with the desired final buffer.

3. *Concentration of protein solutions*

Column effluent fractions and other samples having immunoglobulin concentrations which were too low to be detected by double immunodiffusion or which had to be applied subsequently to Sephadex G-200 or Sepharose 4B columns were concentrated by ultrafiltration For this purpose ultrafiltration in Amicon cells with UM10 or XM300 membranes was used.

C. *Experimental procedures*

The experimental techniques are described here in the same order as they were used in the purification procedure. Pooled normal human serum was used as the starting material.

1. *Purification sequence A*

Part I. *Precipitation methods*

i. *Precipitation with dextran sulfate*: Dextran sulfate (M.W. 560,000) and calcium chloride were added to pooled human serum to final concentrations of 0.5% and 0.09 M, respectively. The mixture was then centri-

fuged at 5,800 x g for 30 min at 4°C in a refrigerated centrifuge (Ivan Sorvall, RC2-B). The precipitate was discarded and the supernatant saved for further processing. The purpose of this procedure was to remove low density lipoproteins (6).

ii. *Precipitation with ammonium sulfate*: A solution of saturated ammonium sulfate (SAS) was prepared by adding 550 g ammonium sulfate to 1,000 ml distilled water. The solution was heated until all of the ammonium sulfate was dissolved, then filtered while hot and cooled to room temperature. The pH was then adjusted to 7.0 with concentrated ammonium hydroxide.

Equal volumes of SAS and supernatant from the dextran sulfate precipitated serum were mixed and the resultant precipitate collected by centrifugation at 5,800 x g for 30 min at 4°C. The precipitate was redissolved in 0.1 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and reprecipitated twice in the same manner with 50% SAS. The last precipitate was redissolved in 0.1 M phosphate buffer, pH 6.0, containing 0.15 M NaCl and dialyzed extensively against the same buffer to remove residual ammonium sulfate from the solution.

iii. *Precipitation with polyethylene glycol-6000 (PEG-6000)*: In preliminary tests, samples of serum were precipitated with different concentrations of PEG-6000, at various pH's. The precipitates obtained were redissolved in 0.05 M phosphate buffer, pH 8.0, and assayed for various classes of immunoglobulins by the double immunodiffusion technique. Proteins remaining in the supernatants were precipitated with

50% ethanol at -10°C (67) and the precipitates collected by centrifugation at -8°C for 30 min at 12,100 x g. These precipitates were also redissolved in 0.05 M phosphate buffer, pH 8.0, and assayed by immunodiffusion. As a result of these experiments a protocol for precipitation with PEG-6000 was established, as follows:

To the protein solution obtained after ammonium sulfate precipitation was added an equal volume of a 13% PEG-6000 solution in 0.1 M phosphate buffer, pH 6.8, containing 0.15 M NaCl. The mixture was left overnight at 4°C and the precipitate collected by centrifugation at 18°C for 30 min at 7,970 x g. The precipiate was redissolved in 0.05 M phosphate buffer, pH 8.0, and applied directly to a DEAE-cellulose column as described below. Residual PEG-6000 was not adsorbed by DEAE-cellulose and was eluted in conjunction with non-adsorbed proteins (67).

Part II. *Chromatography*

i. *Anion-exchange chromatography*: DEAE-cellulose was decanted several times with distilled water and washed with an excess of the following solutions in the order indicated, before use: (a) 0.3 M monopotassium phosphate; (b) 0.5 N sodium hydroxide; (c) 95% ethanol; (d) 0.05 N sodium hydroxide; and (e) distilled water. Usually 500 - 800 ml of each solution was used for each 50 g of DEAE-cellulose. At each stage the DEAE-cellulose was suspended in the solution for 1 hour, then transferred to a Buchner filter funnel and the supernatant removed by vacuum filtration. After each chromatographic run, the DEAE-cellulose was regenerated by the same process, except that the wash with 0.3 M

monopotassium phosphate was omitted. The acidity of the activated resin in distilled water was adjusted to pH 8.0 and the resin was then equilibrated with the starting buffer. The DEAE-cellulose suspension was then transferred to a glass column, allowed to settle and finally packed with air pressure. The column was connected to a flask containing the starting buffer and prior to application of a protein sample, the gel was washed with an amount of starting buffer equal to about four times the volume of the resin bed.

The molarity of the starting buffer was determined by introducing IgM-containing samples into columns of resin in 0.025 M, 0.05 M or 0.075 M phosphate buffer, pH 8.0. The columns were then washed with the same buffer and non-adsorbed protein fractions were assayed for IgM.

Protein solutions obtained after precipitation with 6.5% PEG-6000 were applied to DEAE-cellulose columns in 0.05 M phosphate buffer, pH 8.0, and the columns were washed with the same buffer until the absorbance at 280 nm of the column effluent was less than 0.10. The starting buffer was then replaced with 0.3 M phosphate buffer, pH 4.7, to elute proteins which had been adsorbed by the resin. The adsorbed protein peak was dialyzed against 0.05 M phosphate buffer, pH 8.0, and applied to a second DEAE-cellulose column. Non-adsorbed proteins were again eluted as described above, and a buffer gradient with increasing molarity and decreasing acidity was applied to the column. The buffer gradient was established as follows:

Two 250 ml beakers, a 600 ml beaker and a 1 l beaker were filled with 110 ml 0.05 M phosphate buffer (PB), pH 8.0, 110 ml 0.075 M

PB, pH 6.3, 220 ml 0.1 M PB, pH 5.3, and 330 ml monopotassium phosphate, respectively. The beakers were placed on a four-place magnetic stirrer (Cole-Parmer Instrument Co., Chicago) and connected (in order of decreasing acidity) with glass tubing. The buffer chamber containing 0.05 M phosphate buffer, pH 8.0, was then connected to the DEAE-cellulose column. The buffer in each beaker was stirred continuously to insure that a uniform gradient of increasing molarity and decreasing acidity was obtained. Effluent fractions of 8 ml were collected from the column at a flow rate of 45 ml/hour, using a LKB Ultrovac Automatic Fraction collector, type 7000. The molarity and acidity of each fraction collected was determined by means of a YSI conductivity bridge Model 31 and an E 300 B Metrohm Herisan pH meter, respectively. A previously prepared standard curve relating increasing concentrations of phosphate buffers to conductivity was used to facilitate determination of the molarities of the effluent fractions.

Effluent fractions were assayed for IgM, IgA, IgG, α_2-macroglobulin and ceruloplasmin by qualitative immunodiffusion as described above. IgM-containing fractions were pooled, concentrated and dialyzed against 0.1 M phosphate buffer, pH 7.2, containing 0.5 M NaCl.

ii. *Gel filtration*: Sephadex G-200 and Sepharose 4B were used in gel filtration experiments, as their reported characteristics appeared to be suitable for IgM purification.

Sephadex G-200 was allowed to swell in distilled water for 3 days at room temperature and decanted several times to remove fine

particles which might interfere with the operation of the column. The gel was then equilibrated in 0.1 M phosphate buffer, pH 7.2, containing 0.5 M NaCl and packed in a glass chromatography column with internal dimensions of 5.0 cm x 100 cm and a maximum bed volume of 1,800 ml.

Pre-swollen Sepharose 4B was suspended in 0.1 M phosphate buffer, pH 7.2, containing 0.5 M NaCl, and packed in a glass chromatography column with internal dimensions of 2.6 cm x 100 cm and a bed volume of 530 ml.

A polystaltic pump (Buchler Instruments) was used to maintain a constant flow rate of 26 ml/hour. Samples applied to the Sephadex column usually had a volume of about 25 ml and a total protein concentration of 200 - 300 mg while those applied to the Sepharose column usually had a volume of 12 ml and a total protein concentration of about 100 mg.

The IgM-containing samples obtained from the second DEAE-cellulose column were chromatographed first on Sephadex G-200. Effluent fractions were assayed for IgM and the IgM-containing fractions were then pooled, concentrated and re-chromatographed on the Sepharose 4B column. The IgM-containing fractions from the Sepharose 4B column in which no α_2-macroglobulin or IgA was detected by qualitative immunodiffusion assays were pooled and these constitute the final IgM preparation for this purification sequence.

2. *Purification sequence B*

Part I. *Precipitation methods*

The precipitation techniques described in sequence A above

were carried out to obtain the 6.5% PEG-600 precipitate.

Part II. *Anion-exchange chromatography*

The 6.5% PEG-6000 precipitate was redissolved in 0.05 M phosphate buffer, pH 8.0, and applied to a DEAE-cellulose column. The unadsorbed proteins were eluted with the same buffer, and adsorbed proteins were eluted with 0.3 M phosphate buffer, pH 4.7, and dialyzed against 0.022 M phosphate buffer, pH 7.4, containing 0.1 M NaCl.

Part III. *Second precipitation with PEG-6000*

The dialyzed samples were precipitated with PEG-6000 at a final concentration of 4%. The protein solutions were diluted to a concentration of 1 mg/ml with 0.022 phosphate buffer, pH 7.4, containing 0.1 M NaCl and mixed with an equal volume of 8% PEG in 0.022 phosphate buffer, pH 7.4, containing 0.1 M NaCl. This mixture was left overnight at 4°C, then centrifuged at 39,100 x g for 30 min at 18°C. The precipitate was resuspended in 0.1 M phosphate buffer, pH 7.2, containing 0.5 M NaCl. The redissolved precipitate represented the final product of purification sequence B.

3. *Purification sequence C*

Part I.

Initial procedures in purification sequence C were the same as those in sequence B except that the second PEG-6000 precipitation was carried out at a PEG concentration of 5%. The resultant precipitates were redissolved in 0.1 M phosphate buffer, pH 7.2, containing 0.5 M NaCl.

Part II. *Gel filtration on Sepharose 4B*

The same column and the same conditions as were described for gel filtration with Sepharose 4B in procedure A, were used to chromatograph the redissolved 5% PEG precipitates. Eluted fractions which contained IgM but no detectable α_2-macroglobulin or IgA were pooled and this material constituted the final preparation from procedure C.

Results

Part I. *Precipitation methods*

The purpose of precipitation with dextran sulfate, ammonium sulfate and PEG-6000 was to obtain a protein solution enriched in IgM.

Ammonium sulfate precipitation was carried out at 50% saturation with ammonium sulfate, since preliminary tests, in which samples of serum were precipitated at different concentrations of ammonium sulfate, revealed that at 50% saturation no IgM was found in the supernatant.

Precipitation with PEG-6000 at pH 6.0 seemed most appropriate because it was found that at higher and lower pH's a higher concentration of PEG-6000 was necessary to precipitate IgM. With 6.5% PEG-6000 at pH 6.0 IgM was completely precipitated, while a significant proportion of the IgA and IgG was found in the supernatant.

All precipitation methods were the same in each of the purification sequences described here and the results will therefore be presented separately. The results of these experiments are summarized in Table 1.

The concentrations of total plasma proteins, IgM, IgA and IgG in the pooled serum were found to be 6,000 mg %, 86 mg %, 195 mg % and 1,250 mg %, respectively. Therefore, IgM represented 1.4% of the total serum proteins, while IgA represented 3.2% and IgG represented 20.8%. The percent recovery of total serum proteins, IgM, IgA and IgG, was determined by relating the amounts of these proteins recovered at each step to the amounts present in the pooled serum.

Table 1. Procedure A

Sample	Amount (mg)				% of total proteins			% Recovery of			
	total proteins	IgM	IgA	IgG	IgM	IgA	IgG	total proteins	IgM	IgA	IgG
Serum (100 ml)	6,000	86 99 I.U.	195 116 I.U.	1,250 143 I.U.	1.43	3.25	20.8	100	100	100	100
0.5% Dextran Sulfate precipitation	4,500	80 92 I.U.	190 113 I.U.	1,020 117 I.U.	1.78	4.2	22.6	75	93	97.4	81.6
50% Ammonium Sulfate precipitation	2,450	79.8 91.8 I.U.	160 95 I.U.	980 112 I.U.	3.26	6.5	40	40.8	92.7	82	78.4
6.5% PEG-6000 precipitation	1,820	76.7 88.2 I.U.	118 70 I.U.	700 80 I.U.	4.2	6.46	38.3	30.4	89.2	60.5	56
1st DEAE-cellulose	349	39.5 45.4 I.U.			11.3			5.82	45.9		
2nd DEAE-cellulose	137	30.7 35.3 I.U.	4.6 2.7 I.U.		22.4			2.3	35.7	2.4	
Sephadex G-200	59.5	24.5 28.2 I.U.			41.1			1	28.4		
Sepharose 4B	31.2 19*	15.6 17.9 I.U.			50 82*			0.52	18.1		

* Values obtained with Human Plasma Fraction II as the reference protein

The data in Table 1 suggest that the application of these precipitation techniques resulted in a marked enrichment of IgM, relative to other proteins present in the original serum specimen. These was a decrease of 69.6% in the total amount of protein present, with 39.5% and 44% decreases for IgA and IgG, respectively, but only a 10.8% decrease in the amount of IgM. -Total protein was reduced to a much greater extent than was total immunoglobulin, implying that proteins not belonging to the immunoglobulin class were preferentially removed by these precipitation procedures. A surprising finding was the large loss of proteins as a result of the dextran sulfate precipitation step, since it had been reported that dextran sulfate at 0.5% final concentration specifically precipitated low density β-lipoproteins (6). Since these are normally present in serum at concentrations of only 280 - 440 mg % (80), the 25% reduction in total serum proteins obtained at this stage was unexpected. Precipitation with dextran sulfate reduced the amount of IgG by 20% but did not significantly reduce the IgA content of the sample. Although 7% of the total IgM was also lost at this stage, subsequent precipitations with ammonium sulfate and PEG-6000 had little effect on IgM concentrations, but resulted in significant reductions of IgA and IgG. The 7% loss of IgM on precipitation with dextran sulfate was an undesirable effect, but the coincident elimination of 20% of the total IgG and, more importantly, all of the low density lipoproteins (which interfere with subsequent precipitation steps) was advantageous and this step was considered, therefore, to be an essential part of the IgM purification sequence.

Part II. *Chromatography and selective precipitation*

In procedure A a purification sequence involving ion-exchange chromatography on DEAE-cellulose columns and gel filtration on Sephadex G-200 and Sepharose 4B columns was evaluated. In an attempt to avoid the apparent contamination with α_2-macroglobulin which was observed in the IgM preparation obtained in that manner, a second procedure (B) employing a further precipitation with 4% PEG-6000 was tested, as was a third procedure (C) involving precipitation with 5% PEG-6000, followed by chromatography on Sepharose 4B.

Procedure A

1. *Anion-exchange chromatography*: As described in methods, IgM-containing samples were fractionated on successive DEAE-cellulose columns. The IgM-containing samples were applied to DEAE-cellulose columns pre-equilibrated with 0.05 M phosphate buffer, pH 8.0, since under these conditions all of the IgM in the samples was adsorbed by the resin. With higher ionic strength buffers loss of IgM along with the unadsorbed protein fraction was observed. A 0.05 M phosphate buffer was preferred to buffers of lower molarity because with 0.05 M phosphate buffer less protein was adsorbed by the resin, allowing one to apply a larger sample to the column. When 0.05 M phosphate buffer, pH 8.0, was used as the starting buffer, up to 1,600 mg of protein could be applied to a column with a bed volume (packed) of approximately 100 ml.

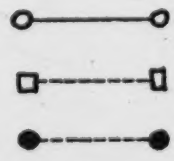
On the first DEAE-cellulose column, which was eluted in a step-wise manner, proteins were separated into two fractions. The

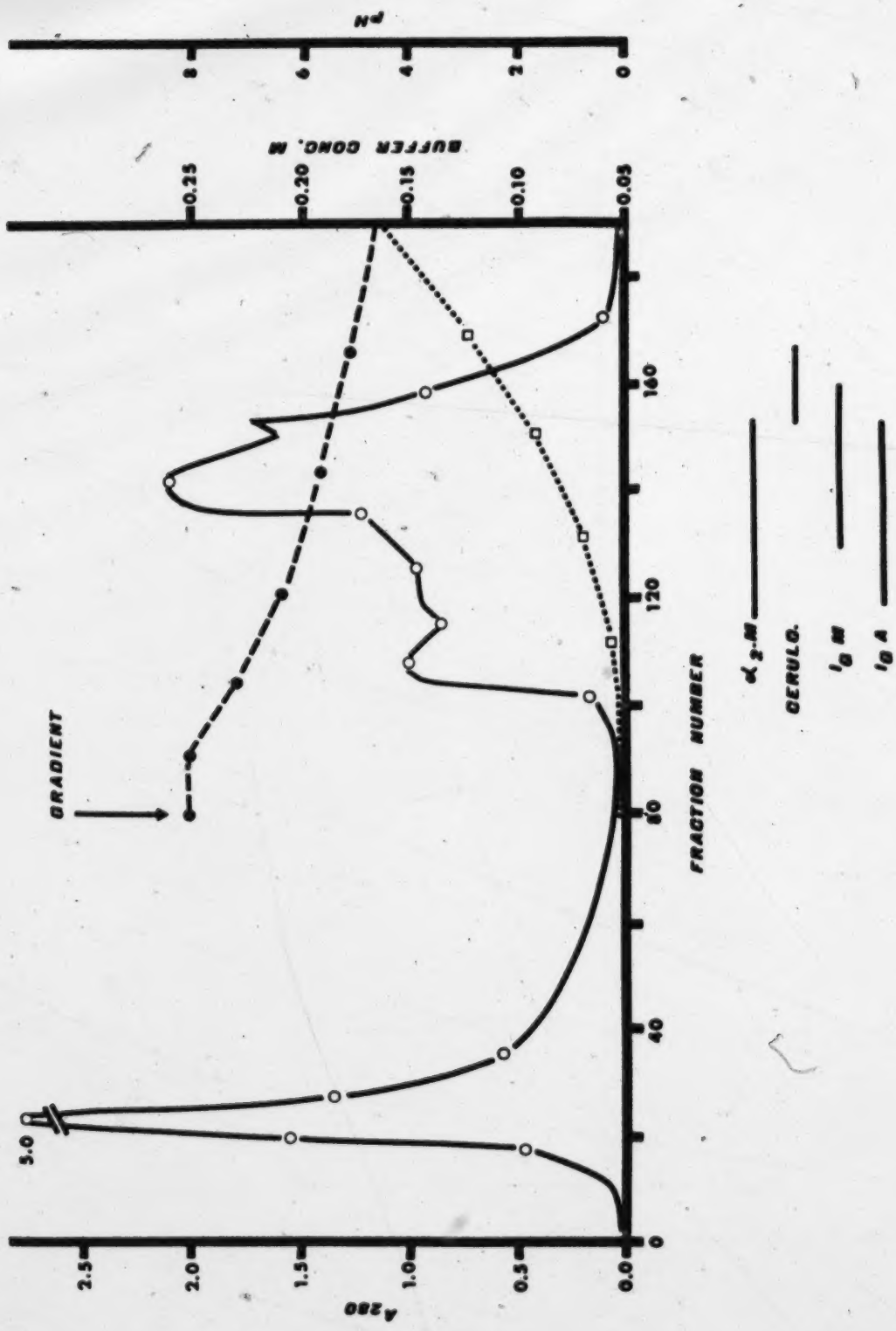
first fraction, representing 45% of the applied protein, contained proteins which were not adsorbed to the resin and which were eluted from the column with 0.05 M phosphate buffer, 8.0. This preparation contained significant amounts of IgA, IgG and α_2-macroglobulin, but no IgM. The second fraction, containing 18.6% of the applied protein, was obtained by eluting the adsorbed proteins from the column with 0.3 M phosphate buffer, pH 4.7. This fraction contained IgM, but was also contaminated with IgG, IgA and α_2-macroglobulin. Replacing the 0.3 M phosphate buffer, pH 4.7, with a buffer gradient with increasing molarity and increasing acidity to elute the adsorbed proteins did not result in an improved separation of IgM from other adsorbed proteins at this stage. In fact, analysis by double immunodiffusion indicated that all fractions eluted in this manner contained detectable amounts of IgM.

On a second DEAE-cellulose column, the "adsorbed protein" fraction from the first column was further fractionated. Approximately 27% of the applied proteins were again recovered in the unadsorbed protein peak, which contained no IgM, while 60.9% of the applied proteins, including most of the IgM, was eluted from the column with a buffer gradient. A typical gradient elution profile is illustrated in Fig. 1. Qualitative immunodiffusion assays were used to determine which of the eluted fractions contained IgM, IgG, α_2-macroglobulin, IgA and ceruloplasmin. IgM began to appear in the effluent when the effluent buffer consisted of 0.07 M phosphate at pH 6.8 and was completely eluted when the effluent buffer composition was 0.11 M phosphate at pH 5.4. Fractions containing α_2- M, IgA, IgG and ceruloplasmin overlapped the

Fig. 1. *Chromatography on DEAE-cellulose*

Approximately 750 mg of protein in 0.05 M phosphate buffer, pH 8.0, were applied to a DEAE-cellulose column with a packed bed volume of 100 ml. Proteins not adsorbing to the resin were eluted with 0.05 M PB. When the absorbance of the effluent at 280 nm declined to less than 0.10, a buffer gradient of increasing molarity and decreasing acidity was started. Fractions of 8 ml each were collected at a flow rate of 75 ml/hr. Individual fractions from the adsorbed peak (second peak) were tested for the presence of IgM, α_2- M, ceruloplasmin and IgA by immunodiffusion. The distribution of these proteins is represented as solid lines under the figure; for example, IgM was found in fractions 128 to 162.




pH
BUFFER CONC. M
GRADIENT
A280
FRACTION NUMBER
α2-M
CERULO.
IgM
IgA

IgM-containing peak (Fig. 1).

Chromatography on DEAE-cellulose resulted in an enrichment of the IgM concentration from 4.2% in the PEG-6000 precipitate to 22.4% of the total proteins in the IgM-containing fractions after ion-exchange chromatography. Moreover, the IgM concentration relative to the IgA concentration increased from 4.2/6.46 (0.65) to 22.7/3.4 (6.6), a ten-fold enrichment relative to IgA (Table 1). The enrichment of IgM relative to IgG was not determined since the IgG concentration in the sample obtained after anion-exchange chromatography was too low to be measured accurately by the methods available. However, immunoelectrophoresis with monospecific anti-human IgG serum suggested that some residual IgG was still present.

The data obtained at various stages during the ion-exchange chromatography procedure and referred to in the text above and in Table 1, indicate that not all of the proteins which were applied to the columns were recovered in the unadsorbed and adsorbed/eluted fractions. Loss of total protein with the first column (step-wise elution) was consistently in the order of 35%, while loss with the second column was less extensive, being in the order of 12%. At the same time there were losses of 54% and 22% of the applied IgM from the first and second DEAE-cellulose columns, respectively. It seemed likely that these losses reflect very tight binding of protein to the ion-exchange resin. Attempts to elute additional protein with buffers containing up to 1.0 M NaCl were only partly successful. In addition, some protein could be eluted when the column was washed with 0.5 M sodium hydroxide. However, the amount of protein recovered by the

combination of these two procedures did not account for all of the lost proteins. Moreover, IgM could not be detected in protein fractions eluted in this manner. It was initially thought that losses of IgM might have resulted, in part, from sample handling at that stage (i.e. concentrating and dialysis). However, this is not now considered to be a reasonable explanation for these large losses since the samples were also subjected to similar procedures at other stages (Sephadex G-200) without experiencing similar losses.

ii. *Gel filtration:* When the IgM-containing fractions obtained after DEAE-cellulose chromatography were chromatographed on Sephadex G-200 columns, proteins were eluted from the column in two separate peaks, the first of which appeared after an elution volume of 495 ml and contained most of the proteins (Fig. 2). Analysis of the eluted fractions by qualitative immunodiffusion indicated that IgM was eluted in the first peak as were α_2-macroglobulin, IgA and a small amount of IgG. Ceruloplasmin and the remaining IgG were found together in the second peak. A third smaller peak was also seen which may have contained some residual albumin, since this protein was still detected by immunoelectrophoresis after DEAE-cellulose chromatography but not after Sephadex G-200 chromatography.

As seen in Table 1, the IgM concentration in the sample obtained following Sephadex G-200 chromatography was enriched by a factor of two. As well, the complexity of the protein mixture, as determined by immunoelectrophoresis, was markedly reduced. Following DEAE-cellulose chromatography several precipitation lines were detected,

Fig. 2. *Chromatography on Sephadex G-200*

The IgM-containing fractions derived from DEAE-cellulose column chromatography were pooled, concentrated and subjected to chromatography on Sephadex G-200. Approximately 260 mg of protein in 25 ml of buffer were applied to a 5.5 cm x 90 cm column of Sephadex G-200. Fractions containing 6,5 ml each were collected at a constant flow rate of 26 ml/hr. The protein content of the fractions is expressed as absorbance at 280 nm and the elution positions of IgM, IgA and α_2- M, as determined by qualitative immunodiffusion, is indicated with solid lines under the figure.

Absorbance at 280 nm

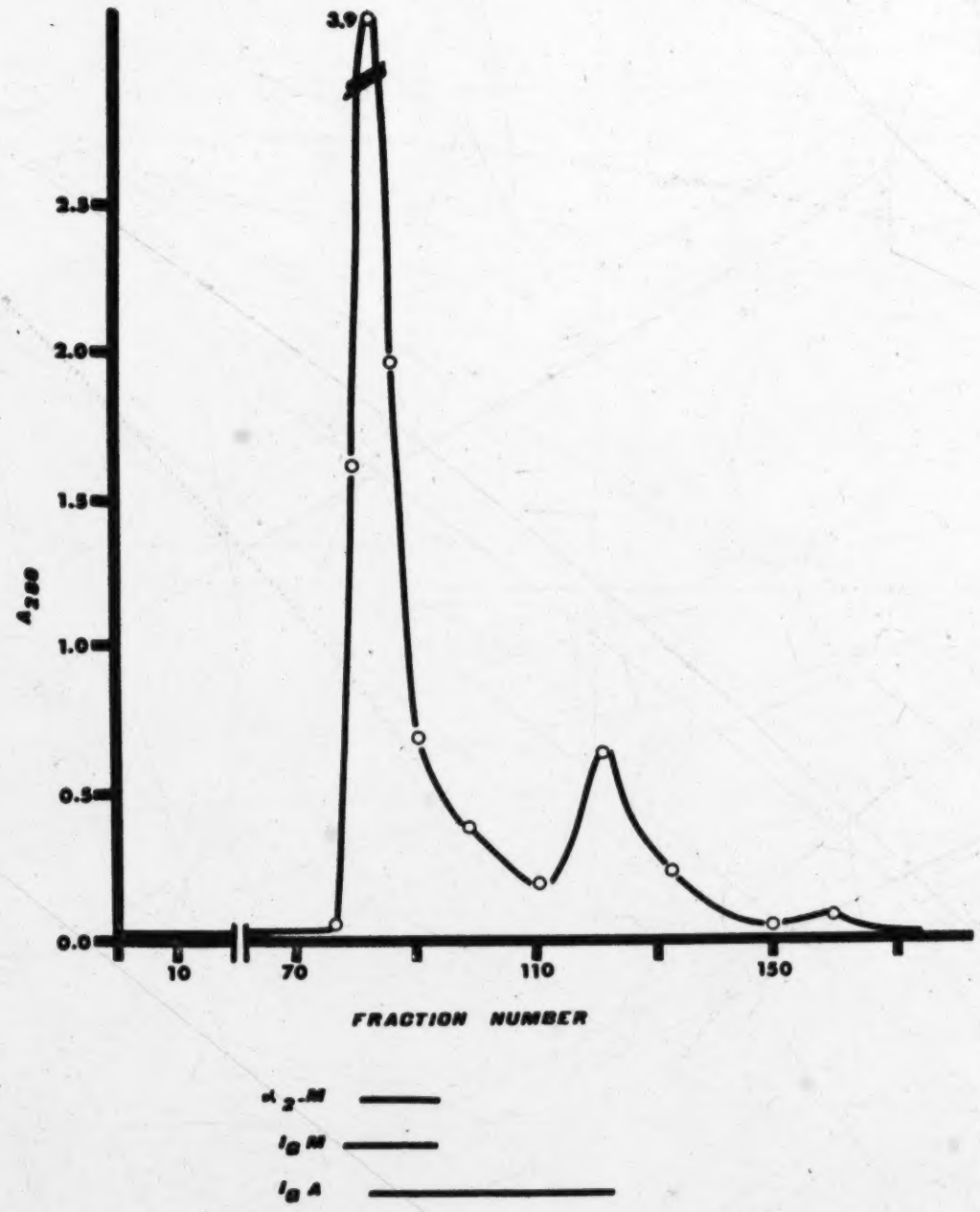
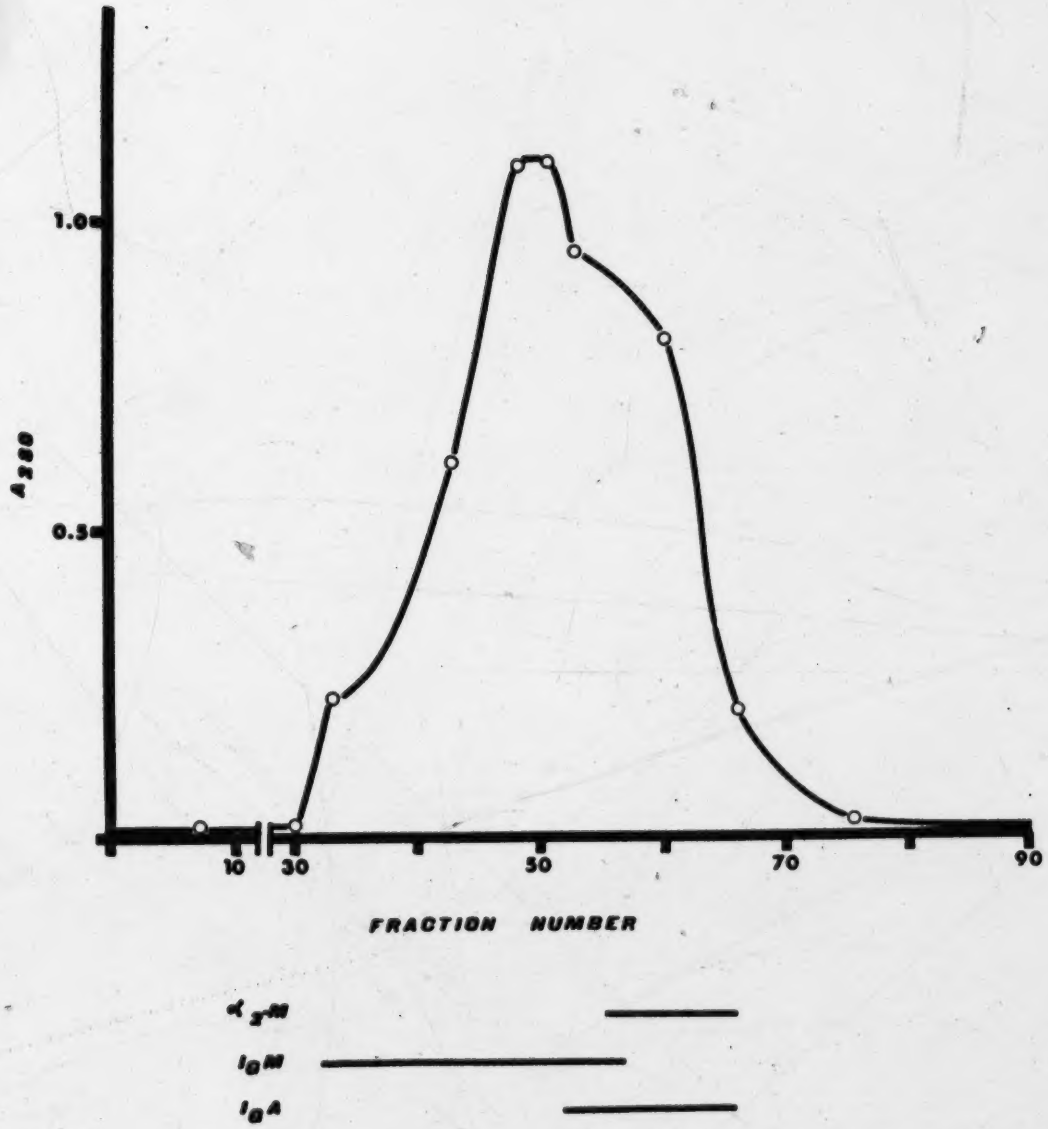

3.9
2.5
2.0
1.5
1.0
0.5
0.0
A_{280}
10
70
110
150
FRACTION NUMBER
α_2-M
I_β M
I_β A

Fig. 3. *Chromatography on Sepharose 4B*

Approximately 100 mg of protein from the IgM-containing fraction obtained after gel filtration on Sephadex G-200 were applied to a Sepharose 4B column (2.5 cm x 100 cm). Fractions of 5.5 ml were collected at a constant flow rate of 26 ml/hr and analyzed by qualitative immunodiffusion for IgM, α_2- M and IgA. The elution position of these three proteins is represented by solid lines under the figure.

Absorbance at 280 nm


1.0
0.5
A_{280}
10
30
50
70
90
FRACTION NUMBER
α_{2}-M
I_{D}M
I_{D}A

while only three lines were observed after Sephadex G-200 chromatography. Immunoelectrophoresis using monospecific anti-human IgG serum suggested that a trace of IgG was also present.

In order to obtain further purification of IgM, the IgM-containing fractions from the Sephadex G-200 column were pooled, concentrated and chromatographed on a Sepharose 4B column. Analysis of the effluent fractions by qualitative immunodiffusion suggested that IgM was largely separated from IgA and α_2-macroglobulin by this procedure. A typical elution profile for Sepharose 4B chromatography is illustrated in Fig. 3.

iii. *Analysis of final IgM preparation*: A total of 15.6 mg of IgM, as determined by quantitative radial-immunodiffusion was recovered following chromatography on Sepharose 4B. This corresponded to an IgM recovery of 18.1%. Total protein recovered, as determined by the Lowry method with bovine serum albumin as the reference protein, was calculated to be 31.2 mg. However, when human plasma fraction II was used as the reference protein, total protein recovery was calculated to be 19 mg. Relative to these values, the amount of IgM recovered represented either 50% or 82% of the total protein recovered, implying that there was either a 50% or an 18% contamination of the final IgM product by other proteins. These values may not reflect the true situation, however, since protein determination and IgM determination were based on different methods. The manner in which IgM concentrations were determined for the purpose of standardizing the quantitative radial-immunodiffusion quantitative assay is unknown, but may have involved

Fig. 4. *Immunoelectrophoretic pattern of IgM preparation from procedure A (#13) and B (#22)*

Antiserum in the upper trough was anti-whole human serum, while the second, third and fourth troughs contained, respectively, anti-IgG, anti-IgA and anti-IgM serum. The upper sample well contained whole human serum and all the other wells contained a sample of the final IgM preparation. Anode was to the left. The analyzed samples had an IgM concentration of 80 mg % in A and 200 mg % in B. Immunoelectrophoresis was carried out as described in Methods.

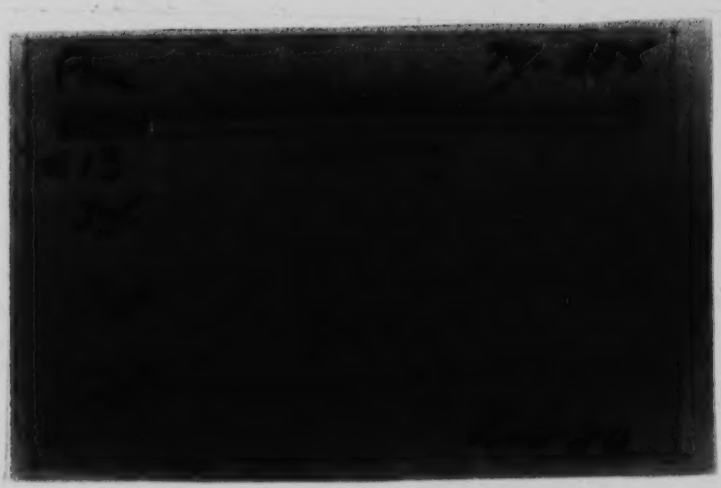
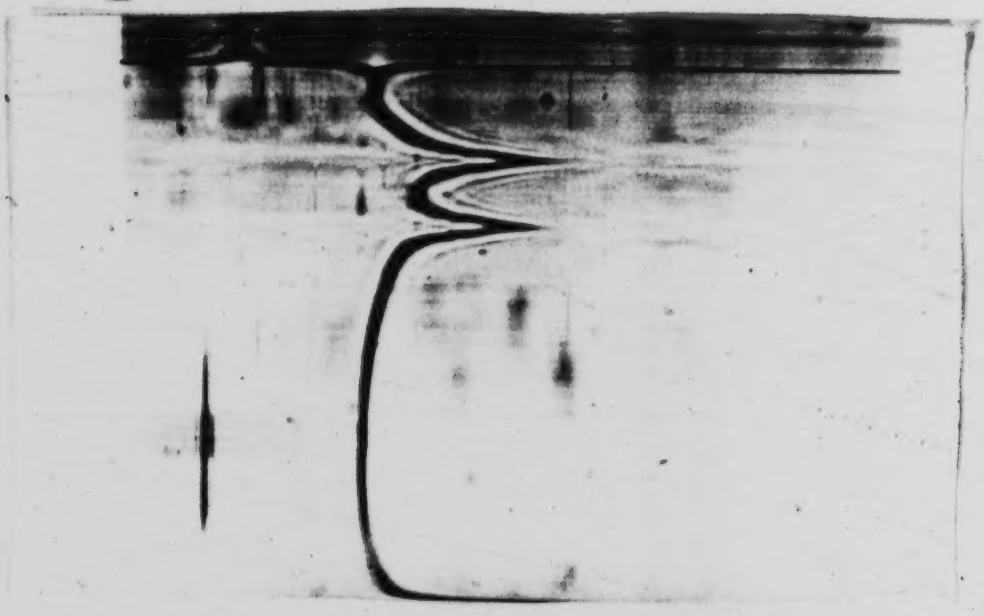


Fig. 5. *Ultracentrifugation-of IgM preparation from procedure A*

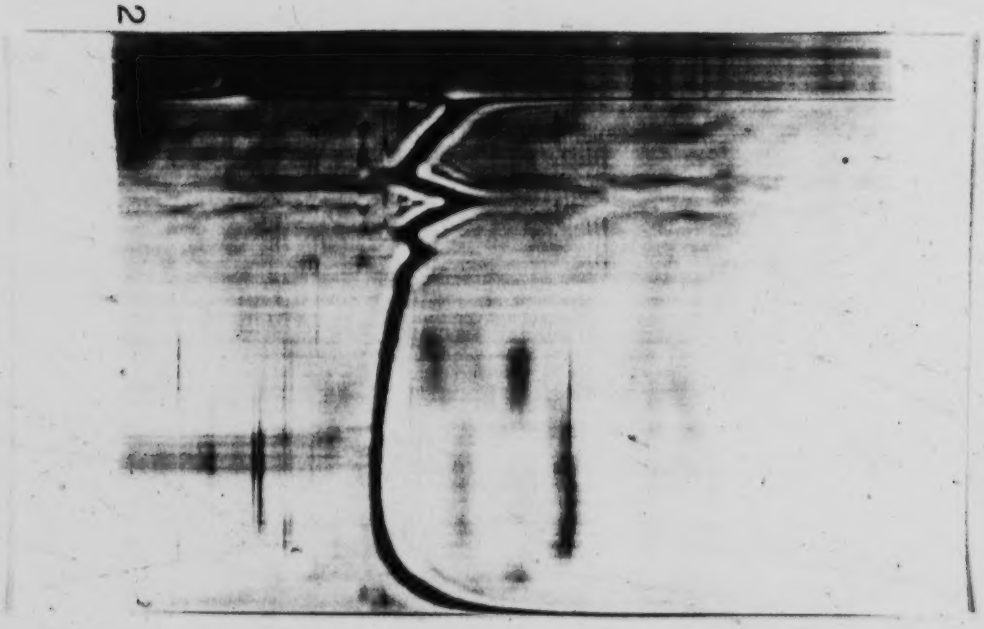
1. Ultracentrifugation pattern of the IgM preparation before dialysis against 6 M urea. Centrifugation was carried out at 20°C in 0.1 M phosphate buffer, pH 7.0, containing 0.1 M NaCl. The protein concentration of the sample was 5 mg/ml.
2. Ultracentrifugation pattern of the IgM preparation after dialysis against 6 M urea. The sample was analyzed at 20°C in 0.1 M phosphate buffer, pH 7.0, containing 0.5 M NaCl. The protein concentration was 4 mg/ml.

Both photographs were taken 12 min after reaching a full speed of 60,000 r.p.m. in a Beckman Model E ultracentrifuge.

1



2



nitrogen determination by the Kjeldahl method. How this might relate to protein concentrations determined by the Lowry method was difficult to assess. Therefore, additional techniques, including quantitative determination of α_2-macroglobulin, as well as immunoelectrophoresis and ultracentrifugation, were used to assess the purity of the final IgM preparation.

Determination of the concentration of α_2-macroglobulin in the final preparation by radial-immunodiffusion, suggested that approximately 3 mg of α_2-macroglobulin was present. This represented 9.6% of the total protein when albumin was taken as the protein standard (or 15.7% when HPF II was used as the standard).

Following immunoelectrophoresis against anti-whole human serum, two precipitation lines were observed, including one which clearly corresponded with IgM and a faint second band which could not be identified (Fig. 4). However, it was thought to be unlikely that the faint line corresponded to α_2-macroglobulin since no precipitation lines were detected with monospecific anti-human α_2-macroglobulin serum. Traces of IgA and IgG were detected, however, with the appropriate monospecific antisera (Fig. 4).

Ultracentrifugation of the final preparation revealed two peaks with observed sedimentation coefficients of 15.96 S and 24.63 S, corresponding to corrected values (S^{w}_{20}) of 17.9 S and 27.7 S (Fig. 5). An indication of a component (or components) with a higher sedimentation coefficient is seen, since a base-line irregularity is evident to the right of the 27.7 S peak. Based on its sedimentation value, the 17.9 S peak may represent IgM, while the 27.7 S peak and the hump following it

may represent polymers or aggregates of IgM, since such components have often been observed in preparations of IgM (58). No discrete components with sedimentation coefficients of less than 17.9 S were detected and no pattern characteristic for lipoproteins was seen. The base-line preceding the major peak showed an initial decay.

The ultracentrifugation pattern described here and illustrated in Fig. 5-2 corresponds to a purified IgM preparation which had been successively dialyzed against 6 M urea and 0.1 M phosphate buffer, pH 7.0, containing 0.5 M NaCl. This further processing of the IgM preparation was carried out in an attempt to reduce the amount of very large IgM aggregates which tend to precipitate since these were observed as rapidly sedimenting material during the first attempt at ultracentrifugation of the IgM preparation. After the early rapid sedimentation of very large IgM aggregates, the ultracentrifugation pattern of an unprocessed samples showed two peaks (Fig. 5-1) with observed sedimentation values of 16.9 S and 28.1 S, corresponding to corrected sedimentation values of 18.06 S and 29.9 S. No decay of the base-line was observed in these runs, suggesting that the initial decay observed in samples which had been dialyzed against 6 M urea was related to this further handling of the IgM preparation.

Procedure B

1. *Anion-exchange chromatography*: Data for enrichment of IgM and recovery of IgM and total protein after chromatography on DEAE-cellulose are outlined in Table II and correspond closely to the values derived after purification sequence A (Table I).

Table 2. Second Part of Procedures B and C

Sample	Amount (mg)				% of total proteins			% Recovery of			
	total proteins	IgM	IgA	IgG	IgM	IgA	IgG	total proteins	IgM	IgA	IgG
DEAE-cellulose	323.4	38.5 44.3 I.U.	11.8 7.0 I.U.		11.9	3.65		5.4	44.7	6.05	
4.0 % PEG-6000 precipitation	41 26.8*	20.3 23 I.U.			49.5 75*			0.68	23.5		
DEAE-cellulose	323.4	38.5 44.3 I.U.	11.8 7.0 I.U.		11.9	3.65		5.4	44.7	6.05	
5.0 % PEG-6000 precipitation	67	27.9 32 I.U.			41.5			1.12	32.4		
Sepharose 4B	29.6 21*	20 23 I.U.			67.6 95.2*			0.5	23.2		

* Values obtained with Human Plasma Fraction II as the reference protein

ii. *Precipitation with 4% PEG*: Since it appeared that a significant amount of α_2-macroglobulin contaminated the IgM-containing preparation obtained by purification sequence A, an additional PEG-6000 precipitation step was included following DEAE-cellulose chromatography in a further attempt to separate IgM from α_2-macroglobulin.

The rationale for this approach was based on the experiments reported by Iverius and Laurent (40), who found that at pH 7.4 in 0.022 phosphate buffer containing 0.1 M NaCl, IgM precipitated from plasma diluted to 10 mg protein/ml at a PEG-6000 concentration of 3%, while α_2-macroglobulin precipitated at 8% PEG-6000. IgA and IgG were reported to precipitate at 12% and 8% PEG-6000, respectively.

Portions of the IgM-containing sample obtained after chromatography on DEAE-cellulose, were precipitated with concentrations of PEG ranging from 3% to 7% at the same pH and ionic strength but at a lower protein concentration (1 mg/ml) than in Iverius and Laurent's experiments (40). Supernatants and precipitates were assayed for IgM, α_2-macroglobulin, IgA, IgG and ceruloplasmin. The results of these experiments differed from Iverius and Laurent's results, since it was found that IgM began to precipitate at 3% PEG but was not completely precipitated until 7% PEG was used. IgA and α_2-macroglobulin began to precipitate at 5% PEG, but were still found in the supernatant at 7% PEG concentration. Ceruloplasmin did not precipitate at these concentrations of PEG-6000.

Precipitation of IgM-containing samples from DEAE-cellulose columns with 4% PEG-6000 was subsequently used to separate IgM from α_2-macroglobulin, IgA and ceruloplasmin.

iii. *Analysis of final IgM preparation (Procedure B)*: Only one precipitation line, corresponding to IgM, was detected following immunoelectrophoresis of the sample against anti-whole human serum. However, when monospecific anti-IgM, anti-IgA and anti-IgG sera were used, faint precipitation lines indicative of the presence of traces of IgA and IgG in the sample were seen (Fig. 4).

Quantitative determinations indicated that 47.3% of the IgM in the sample was lost as a result of precipitation with 4% PEG, although the overall recovery of IgM from pooled serum was still 23.5% (Table II) compared to the 18.1% recovery obtained by procedure A. The total protein recovered as determined by the Lowry method, was 41 mg when bovine serum albumin was used as the reference protein or 26.8 mg with HPF II as the reference protein. The IgM recovered, as determined by quantitative radial-immunodiffusion assay, was 20.3 mg corresponding to either 49.5% or 75% of the total protein, depending on which reference protein is used in making the calculation. These values implied either a 50% or 25% contamination with non-IgM proteins in the final preparation obtained by this method.

Quantitative determination by radial-immunodiffusion assay indicated that only 1.13 mg of α_2-macroglobulin was present, representing either 2.7% or 4.2% of the total protein in that preparation.

The results of the ultracentrifugation experiments were similar to those pertaining to the IgM product from procedure A. After treatment of the IgM preparation with 6 M urea, two peaks with observed sedimentation coefficients of 16.37 S and 24.66 S, corresponding to corrected sedimentation coefficients (S^{w}_{20}) of 18.4 and 27.7

were observed (Fig. 8).

Procedure C

i. *Precipitation with 5% PEG and gel filtration on Sepharose 4B*: A portion of the same sample from DEAE-cellulose as was used in procedure B was precipitated with 5% PEG.

Quantitative assays indicated that only 27.8% of the added IgM was lost by precipitation with 5% PEG as compared to the 47.3% loss following precipitation with 4% PEG. Precipitation with 5% PEG increased the IgM concentration from 11.9% to 41.5% of the total protein (relative to bovine serum albumin as the reference protein) (Table II). Immunoelectrophoresis against anti-whole human serum indicated two distinct precipitation lines in addition to the precipitation line corresponding to IgM. Both IgA and IgG were detected with monospecific anti-IgA and anti-IgG sera (Fig. 6).

The 5% PEG precipitate was then chromatographed on a Sepharose 4B column in an attempt to separate IgM from IgG, IgA and α_2-macroglobulin. The relative elution positions of these components were determined by qualitative immunodiffusion assays and were shown to be similar to the results obtained after chromatography on Sepharose 4B in procedure A (Fig. 3). Fractions containing only IgM were pooled and concentrated.

ii. *Analysis of final IgM preparation*: The amount of IgM lost during precipitation with 5% PEG was less than with 4% PEG, but the final recovery of IgM was the same as in procedure B (23.3%) since there was

Fig. 6. *Immunoelectrophoretic pattern of IgM-containing solution obtained after precipitation with 5% PEG*

Antisera were applied to the antisera troughs (#12) in the same order as described in Fig. 4. The upper antigen well contained whole human serum; the second, third and fourth antigen wells contained samples of the protein solution obtained after 5% PEG precipitation. The IgM concentration in the samples was 126 mg %.

an additional loss of IgM (9.1%) during chromatography on Sepharose 4B (Table II).

IgM constituted 67.6% or 95.2% and α_2-macroglobulin constituted 3.2% or 5.2% of the total protein, relative to bovine serum albumin or HPF II as the reference proteins, respectively.

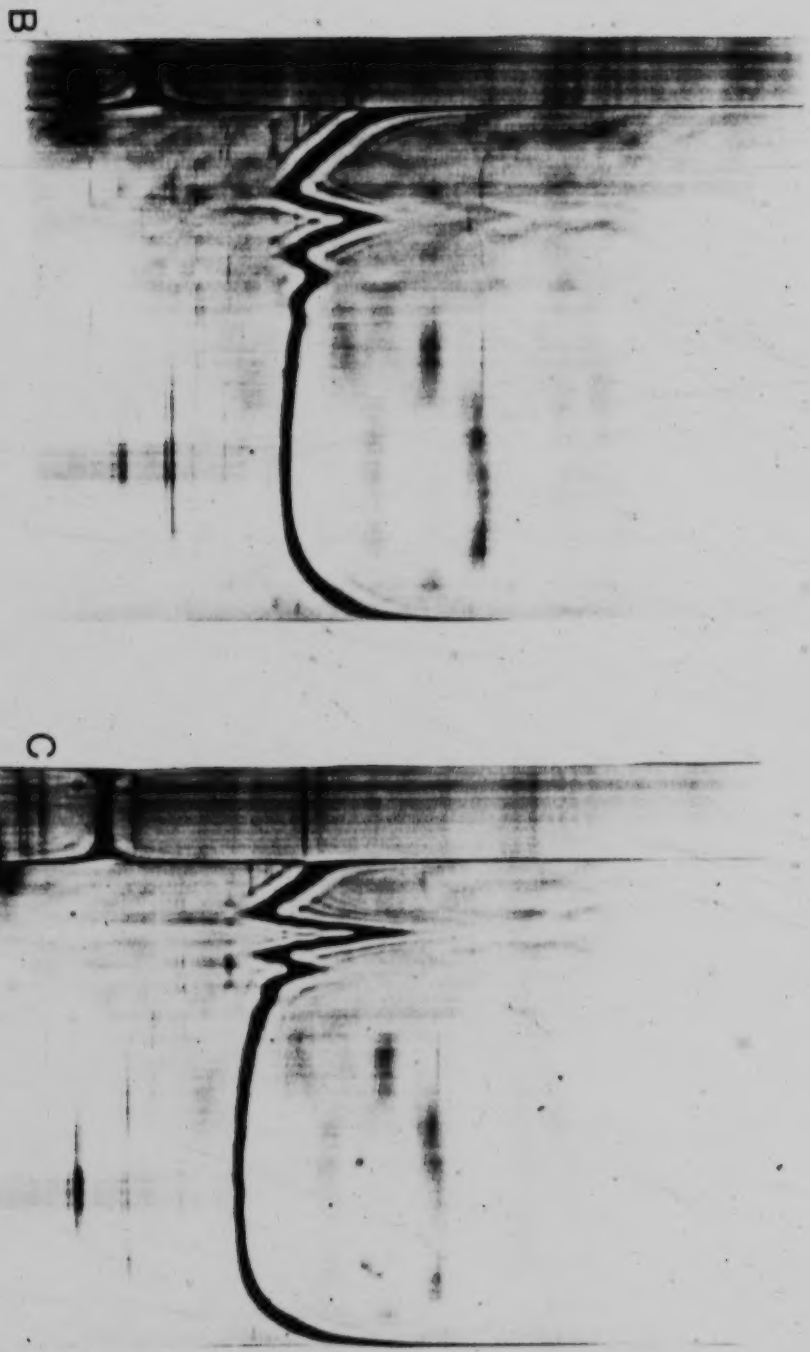
The immunoelectrophoretic pattern (Fig. 7) was similar to that obtained with the final preparation from procedure B, and the ultracentrifugation pattern was similar to that for the final product from procedure A. After dialysis of the IgM preparation against 6 M urea, the observed sedimentation coefficients for the two peaks were 16.96 S and 25 S corresponding to corrected values (S^w_{20}) of 18.77 S and 28.2 S (Fig. 8).

Fig. 7. *Immunoelectrophoretic pattern of IgM preparation from procedure C (#23)*

Antisera were applied to the antisera troughs as previously described. The upper antigen well contained whole human serum, while the other antigen wells contained samples of the final IgM preparation from procedure C. This preparation had an IgM concentration of 200 mg %.

Fig. 8. *Ultracentrifugation of the IgM preparations of procedures B and C*

The samples were analyzed as described in Fig. 5-2 and in Methods. Photographs were taken at 16 min (B) and 12 min (C) after reaching full speed.


B
C

DISCUSSION

Three procedures for large scale purification of IgM from normal human serum were evaluated.

The final IgM recoveries after following procedures A, B and C were 18.1%, 23.5% and 23.3%, respectively. It may be suggested that the recovery of IgM could be increased significantly if an alternative to DEAE-cellulose column chromatography was available, since very large losses were incurred at that stage in the procedure. Using different approaches, Vaerman *et al.* (90) obtained 30 to 40% recovery and Chaplin *et al.* (7) obtained a 25% recovery. However, their procedures involved preparative ultracentrifugation, which is better adapted to microscale preparations. Large scale IgM purification has been attempted in some clinical studies. Wickerhauser and Hao (92) obtained an IgM recovery of 25% with a procedure consisting of PEG precipitation, zinc sulfate precipitation, chromatography on Sephadex G-200 and ammonium sulfate precipitation. However, purity of the IgM preparation was not one of their major concerns and a 55% contamination of the IgM preparation with other serum proteins was observed.

In contrast the purpose of the experiments reported here was to develop a large scale preparative procedure for obtaining very high purity IgM from normal human serum in reasonable yields.

Among the three procedures tested in our experiments, procedure B seemed most suitable for large scale preparation, since it did not involve chromatography on Sephadex G-200 or Sepharose 4B. These chromatographic techniques are somewhat limiting in terms of the

amount of protein that can be chromatographed at once without sacrificing resolution. Nevertheless, procedures A and C could also be applied for preparative purposes, since the chromatographic steps are introduced at the end of the purification sequence and at that stage the problem of handling large amounts of protein is reduced.

In fact, the purity of the final IgM preparation from procedure C appeared to be somewhat superior to the products of the other two procedures, since the final IgM product from procedure C was 95.2% pure, while the IgM preparations from procedures A and B were 82% and 75% pure, respectively. Those values apply to protein concentrations determined by the Lowry method (52) with human plasma fraction II as the reference protein. It was assumed that with HPF II a more correct estimate of the protein concentration would be obtained than would be obtained if albumin was used as the reference protein, since the chemical composition of IgM is more closely related to HPF II (gamma globulins) than to albumin.

Calculations of the purity of these preparations, based on determinations of total protein (by the Lowry method) and IgM concentrations (by quantitative radial immunodiffusion) were, for the most part, in reasonable agreement with indications of purity obtained by other methods, although some unexplained discrepancies were noted with the products from procedures B and C. The immunoelectrophoretic and ultracentrifugation patterns of the final preparations from procedures B and C were very similar and their α_2-macroglobulin content, as determined by quantitative radial immunodiffusion was almost identical. Yet, the amount of contaminating proteins was calculated to be 25% in

procedure B and 4.8% in procedure C. One possible explanation for the observed discrepancies may involve the quantitative radial immunodiffusion assay for IgM. Variations in that assay could result in the calculation of an inordinately low value for IgM concentrations so that the concentrations of contaminating proteins would appear greater than they actually were. The final products from procedures B and C showed, after immunoelectrophoresis, only one precipitin line which corresponded to IgM, while the final preparation from procedure A showed two precipitin lines, one corresponding to IgM and one to an unidentified protein.

Contamination with α_2-macroglobulin was also far more pronounced after procedure A (3 mg as compared to 1.1 mg for B and C). This suggests that selective precipitation with 4% PEG, as in procedure B, or precipitation with 5% PEG in combination with gel filtration, as in procedure C, was particularly effective in eliminating α_2-macroglobulin.

With regard to contamination with IgA and IgG, no basic differences were observed between the three procedures. Both immunoelectrophoresis and qualitative immunodiffusion assays suggested that small traces of those immunoglobulins seemed to be present. However, quantitative determinations could not be carried out since the concentrations of those immunoglobulins were too low to be determined by the radial immunodiffusion technique. As well, ultracentrifugation patterns did not show either 7 S components or other components with sedimentation rates known to correlate with IgA. It might be suggested that cross reactions between the anti-IgA

and anti-IgG antisera and IgM could give a false impression that small amounts of IgA and IgG were still present in the IgM preparation. This would occur if all of the anti-IgM activity has not been absorbed during preparation of these monospecific antisera, as well as if true cross reactions were occurring. Alternatively, small amounts of residual IgA and IgG may indeed have been present, although this was not apparent in the ultracentrifugation analysis.

The overall pattern of the sedimentation profile for IgM obtained by procedure A (Fig. 5), showed two peaks with corrected sedimentation coefficients (S_w^{20}) of 18.065 and 29.95. In an attempt to reduce the amount of IgM aggregates, the sample was dialyzed against 6 M urea. As a result the amplitude of the polymer peak relative to the monomer peak was reduced, and the sedimentation coefficients were slightly decreased (17.9 S and 27.7 S). A very small third peak with a higher sedimentation rate than 27.7 S also appeared. Base line decay, possibly related to bacterial contamination or denaturation of the sample was observed in samples which had been treated in this manner as well. The ultracentrifugation patterns of all three IgM preparations, after dialysis against 6 M urea, were similar. The corrected sedimentation coefficients (S_{20}^w) of the major components of the three IgM preparations varied from 17.9 S to 18.7 S, while those of the minor heavier components were almost equal, averaging 27.4 S. These values are in very good agreement with those reported for IgM by Filitti-Wurmser *et al.* (23) and numerous other investigators.

After a review of all of the data available, it was concluded that procedure C be recommended as being most satisfactory for large

scale purification of normal human IgM.

A continuing problem which was noted throughout the course of this investigation related to the specificity of commercial antisera which are available for identifying and quantitating serum proteins. Although the final IgM products obtained by employing purification procedures B and C exhibited only single precipitin bands when tested against anti-whole human serum and showed characteristic profiles for purified IgM on ultracentrifugation, nevertheless faint precipitin bands were also detected in those preparations when monospecific anti-IgA and anti-IgG antisera were employed. There are two possible explanations for these observations: either small amounts of IgA and IgG were present in the IgM preparations, or the commercial anti-IgA and anti-IgG antisera were not truly monospecific. Since the ultracentrifugation analysis did not indicate the presence of discrete components with sedimentation coefficients less than 15 S (i.e. in the range expected for IgG and IgA), it was concluded that the specificity of anti-IgA and anti-IgG antisera might be subject to question.

The difficulties inherent in obtaining truly monospecific antisera by commercial precipitation methods are obvious, since problems can be encountered in attaining equivalence points for each of the variety of different antigen-antibody complexes which will be formed when the antiserum is mixed with the preparation of precipitating antigens. The resultant "monospecific" antiserum is likely to contain residual, undesired antibody activities when that approach is taken. An alternative approach is to include in this procedure the use of

solid phase adsorption techniques wherein a purified antigen is immobilized on an insoluble support and this adsorbent is used to isolate the desired antibody from the antiserum. The difficulties in this case arise at a preliminary stage - that is, in obtaining the purified antigen preparation. However, once that problem is overcome, as it can be (although with some difficulty in certain instances) by the application of classical protein purification techniques, a two-stage procedure involving precipitation with soluble antigens followed by adsorption on specific solid phase adsorbents could be used for the commercial production of high quality monospecific antisera.

Because of the uncertainty as to the true specificities of the antisera used in this investigation, it is not possible to state unequivocally either that the IgM preparations obtained by procedure C were pure, or that they were in fact contaminated with small amounts of other serum proteins. What can be concluded, however, is that a convenient large scale preparative procedure for the isolation of normal human IgM of relatively high purity in 20-25% yields has been derived. More reliable immunological reagents must be available before a more precise definition of the purity of these IgM preparations can be given.

BIBLIOGRAPHY

1. AGRAWAL, B.B.L. and GOLDSTEIN, I.J., 1967. Protein-carbohydrate interaction. VI. Isolation of concanavalin A by specific adsorption on cross-linked dextran gels. Biochim. Biophys. Acta *147*: 262-271.

2. ASPBERG, K. and PORATH, J., 1970. Group-specific adsorption of glycoproteins. Acta Chem. Scand. *24*: 1839-1841.

3. BECKER, W. and STÖRIKO, K., 1970. Immunological determination of immunoglobulins: normal values and preparation of a standard. In: *Progress in Immunological Standardization*, Vol. 4, pp. 129-135 (ed. R.M. Remagey, A. de Barbieri, W. Hennessen, D. Ikič and F.T. Perkins) Karger, Basel, München, New York.

4. BENGTSSON, S. and PHILIPSON, L., 1964. Chromatography on pearl-condensed agar. Biochim. Biophys. Acta *79*: 399-406.

5. BÜRGIN-WOLFF, A., HERNANDEZ, R. and JUST, M., 1971. Separation of Rubella IgM, IgA and IgG antibodies by gel filtration on agarose. Lancet: 1278-1280.

6. BURSTEIN, M. and SAMAILLE, J., 1958. Sur une nouvelle méthode de dosage du cholestérol lié aux α- et aux β-lipoprotéines du sérum. Clin. Chim. Acta *3*: 320-327.

7. CHAPLIN, M., COHEN, S. and PRESS, E.M., 1965. Preparation and properties of the peptide chains of normal human 19 S γ-globulin (IgM). Biochem. J. *95*: 256-261.

8. CHESEBRO, B. and SVEHAG, S.-E., 1968. Precipitation of human serum proteins by polyethylene glycol. Clin. Chim. Acta *20*: 527-529.

9. CHESEBRO, B., BLOTH, B. and SVEHAG, S.-E., 1968. The ultra-structure of normal and pathological IgM immunoglobulins. J. Exp. Med. *127*: 399-409.

10. CHESEBRO, B. and SVEHAG, S.-E., 1969. Biologic activity and morphology of three types of rabbit IgM antibody subunits. J. Immunol. *102*: 1064-1073.

11. CHUN, P.W., FRIED, M. and ELLIS, E.F., 1967. Use of water-soluble polymers for the isolation and purification of human immuno-globulins. Anal. Biochem. *19*: 481-497.

12. DAVIE, J.M. and OSTERLAND, C.K., 1968. Chemical characterization of glycopeptides from human γM-globulins. J. Exp. Med. *128*: 699-714.

13. DEUTSCH, H.F. and MORTON, J.I., 1958. Human serum macroglobulins and dissociation units. I. Physicochemical properties. J. Biol. Chem. *231*: 1107-1118.

14. DONNELLY, E.H. and GOLDSTEIN, I.J., 1970. Glutaraldehyde-insolubilized concanavalin A: an adsorbent for the specific isolation of polysaccharides and glycoproteins. Biochem. J. *118*: 679-680.

15. EDELMAN, G.M. and GALL, W.E., 1969. The antibody problem. In: *Annual Review of Biochemistry*, Vol. 38, pp. 415-466 (ed. E.E. Snell, P.D. Boyer, A. Meister and R.L. Sinsheimer) Annual Reviews, Inc.

16. EDSALL, J.T., 1947. The plasma proteins and their fractionation. In: *Advances in Protein Chemistry*, Vol. 3, pp. 383-479 (ed. Anson, M.L. and Edsall, J.T.) Academic Press Inc., New York.

17. FAHEY, J.L., McCOY, P.F. and GOULIAN, M., 1958. Chromatography of serum proteins in normal and pathological sera. J. Clin. Invest. *37*: 272-284.

18. FAHEY, J.L. and HORBETT, A.P., 1959. Human gamma globulin fractionation on anion exchange cellulose columns. J. Biol. Chem. *234*: 2645-2651.

19. FAHEY, J.L., 1962. Heterogeneity of γ-globulins. In: *Advances in Immunology*, Vol. 2, pp. 41-109 (ed. W.H. Taliaferro and J.H. Humphrey) Academic Press, New York.

20. FAHEY, J.L. and McLAUGHLIN, C., 1963. Preparation of antisera specific for 6.6 S γ-globulins, β_2 A-globulins, γ_1-macroglobulins, and for type I and II common γ-globulin determinants. J. Immunol. *91*: 484-497.

21. FAHEY, J.L. and LAWRENCE, M.E., 1963. Quantitative determination of 6.6 S γ-globulins, β_2 A-globulins and γ_1-macroglobulins in human serum. J. Immunol. *91*: 597-603.

22. FAHEY, J.L. and TERRY, E.W., 1967. Ion-exchange chromatography and gel filtration. In: *Handbook of Experimental Immunology*, pp. 19-43 (ed. D.M. Weir) Blackwell Scientific Publications, Oxford and Edinburgh.

23. FILITTI-WURMSER, S., GENTOU, C. and HARTMANN, L., 1964. Existence de deux varietés 17 S ou 18.3 S de γ_1M et d'une augmentation de globulines 6.6 S sériques dans la maladie de Waldenström. Rev. Fr. Etud. Clin. Biol. *9*: 398-410.

24. FIREMAN, P., VANNIER, W.E. and GOODMAN, H.C., 1963. The association of skin-sensitizing antibody with the β_2 A-globulins in sera from Ragweed-sensitive patients. J. Exp. Med. *117*: 603-619.

25. FIREMAN, P., VANNIER, W.E. and GOODMAN, H.C., 1964. Immunochemical studies of human serum fractionated by gel filtration with Sephadex G-200. Proc. Soc. Exp. Biol. Med. *115*: 845-849.

26. FISCHER, L., 1969. Gels for chromatography. In: *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 1, pp. 175-204 (ed. T.S. Work and E. Work) North-Holland Publishing Co., Amsterdam-London.

27. FLODIN, P., 1961. Methodological aspects of gel filtration with special reference to desalting operation. J. Chromatogr. *5*: 103-115.

28. FLODIN, P. and KILLANDER, J., 1962. Fractionation of human serum proteins by gel filtration. Biochim. Biophys. Acta *63*: 403-410.

29. FRANKLIN, E.C. and FRANGIONE, B., 1967. Two serologically distinguishable subclasses of μ chains of human macroglobulins. J. Immunol. *99*: 810-813.

30. FROMMEL, D., G.W. LITMAN, S.L. CHARTRAND, U.S. SEAL and R.A. GOOD, 1971. Carbohydrate composition in the evolution of immunoglobulins. Immunochemistry *8*: 573-577.

31. GELOTTE, B., FLODIN, P. and KILLANDER, J., 1962. Fractionation of human plasma proteins by gel filtration and zone electrophoresis or ion-exchange chromatography. Arch. Biochem. Biophys. Suppl.*1* : 319-326.

32. GOLDSTEIN, I.J., SO, L.L., YANG, Y. and CALLIES, G.C., 1969. Protein-carbohydrate interaction. XIX. The interaction of concanavalin A with IgM and the glycoprotein phytohemagglutinins of the Waxbean and the soybean. J. Immunol. *103*: 695-698.

33. HANSSON, U.B. and NILSSON, E., 1973. Electroimmunoassay of human immunoglobulins M of different charges. J. Immunol. Methods *2*: 221-228.

34. HARRIS, H. and ROBSON, E.B., 1963. Precipitin reactions between extracts of seeds of concanavalin ensiformis (Jack bean) and normal and pathological serum proteins. Vox. Sang. *8*: 348-355.

35. HEREMANS, J.F. and HEREMANS, M.-Th., 1961. Studies on "abnormal" serum globulins (M-components) in myeloma, macroglobulinaemia and related diseases. Acta Med. Scand., Suppl. *367*: 27-59.

36. HJERTÉN, S., 1962. "Molecular sieve" chromatography on polyacrylamide gels, prepared according to a simplified method. Arch. Biochem. Biophys., Suppl. *1*: 141-151.

37. HJERTÉN, S., 1964. The preparation of agarose spheres for chromatography of molecules and particles. Biochim. Biophys. Acta *79*: 393-398.

38. HJERTÉN, S., 1967. Molecular and particle sieving (chromatographic and electrophoretic) on agarose and polyacrylamide gels. In: *Prot. Biol. Fluids, 14th Colloqu. Bruges*, pp. 553-561 (ed. Peeters, H.) Elsevier Publishing Co., Amsterdam-London-New York.

39. HÖGMAN, C.F., KILLANDER, J. and JOHANSSON, S.H.O., 1964. Identification of immunoglobulins with antibody activity fractionated by gel filtration. Vox. Sang. *9*: 10-13.

40. IVERIUS, P.H. and LAURENT, T.C., 1967. Precipitation of some plasma proteins by the addition of dextran or polyethylene glycol. Biochim. Biophys. Acta *133*: 371-373.

41. JOHANSSON, S.G.O., 1968. Serum IgND levels in healthy children and adults. Int. Arch. Allergy Appl. Immunol. *34*: 1-8.

42. JOHNSON, P. and MILLER, J.M., 1970. Studies on Waldenström macroglobulins. I. Hydrodynamic properties of IgM, its subunits and heavy polypeptide chains. Biochim. Biophys. Acta *207*: 297-307.

43. JUCKES, I.R.M., 1971. Fractionation of proteins and viruses with polyethylene glycol. Biochim. Biophys. Acta *229*: 535-546.

44. KILLANDER, J. and FLODIN, P., 1962. Fractionation of serum proteins by gel filtration. Vox. Sang. *7*: 113.

45. KILLANDER, J. and HÖGMAN, C.F., 1963. Fractionation of human blood group antibodies by gel filtration. Scand. J. Clin. Lab. Invest. *15*: 130-138.

46. KILLANDER, J., 1964. Fractionation of antibodies of 19 S, 7 S and "intermediate" types by gel filtration and ion exchange chromatography or preparative electrophoresis. In: *Prot. Biol. Fluids, 11th Colloqu. Bruges*, pp. 446-449 (ed. Peeters, H.) Elsevier Publishing Co., Amsterdam-London-New York.

47. KILLANDER, J., BENGTSSON, S. and PHILIPSON, L., 1964. Fractionation of human plasma macroglobulins by gel filtration on pearl condensed agar. Proc. Soc. Exp. Biol. Med. N.Y. *115*: 861-865.

48. LAURENT, T.C., 1963. The interaction between polysaccharides and other macromolecules. 5. The solubility of proteins in the presence of dextran. Biochem. J. *89*: 253-257.

49. LAURENT, T.C. and KILLANDER, J., 1964. A theory of gel filtration and its experimental verification. J. Chromatogr. *14*: 317-330.

50. LEON, M.A., 1967. Concanavalin A reaction with human normal immunoglobulin G and myeloma immunoglobulin G. Science *158*: 1325-1326.

51. LLOYD, K.O., 1970. The preparation of two insoluble forms of the phytohemagglutinin concanavalin A., and their interactions with polysaccharides and glycoproteins. Arch. Biochem. Biophys. *137*: 460-468.

52. LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. *193*: 265-275.

53. MANCINI, G., VAERMAN, D.-P., CARBONARA, A.O. and HEREMANS, J.F., 1964. A single-radial-diffusion method for the immunological quantitation of proteins. In: *Prot. Biol. Fluids, 11th Colloqu. Bruges*, pp. 370-373 (ed. Peeters, H.) Elsevier Publishing Co., Amsterdam-London-New York.

54. MANDEMA, E., VAN DER SCHAAF, P.C. and HUISMAN, T.H., 1955. Investigations on the amino acid composition of a macroglobulin and a cryoglobulin. J. Lab. Clin. Med. *45*: 261-269.

55. MARTIN, N.H., 1960. Macroglobulinaemia. Q. J. Med. *29*: 179-197.

56. McKELVEY, E.M. and FAHEY, J.L., 1965. Immunoglobulin changes in disease: quantitation on the basis of heavy polypeptide chains, IgG (γG), IgA (γA) and IgM (γM) and of light polypeptide chains, type K (I) and type L (II). J. Clin. Invest. *44*: 1778-1787.

57. METZGER, H., 1969. Myeloma proteins and antibodies. Am. J. Med. *47*: 837-843.

58. METZGER, H., 1970. Structure and function of γM macroglobulin. In: *Advances in Immunology*, Vol. 12, pp. 57-116 (ed. Dixon, F.J. and Kunkel, H.G.) Academic Press, New York and London.

59. MILLER, F. and METZGER, H., 1965. Characterization of a human macroglobulin. I. The molecular weight of its subunit. Biol. Chem. *240*: 3325-3333.

60. MÜLLER-EBERHARD, H.J. and KUNKEL, H.G., 1956. The carbohydrate of γ-globulin and myeloma proteins. J. Exp. Med. *104*: 253-269.

61. NAKAMURA, S., TANAKA, K. and MURAKAWA, S., 1960. Specific protein of legumes which reacts with animal proteins. Nature *188*: 144-145.

62. OUCHTERLONY, Ö., 1953. Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. Acta Pathol. Microb. Scand. *32*: 231-240.

63. OLESEN, H., MANSA, B. and LIND, K., 1964. Characterization of cold agglutinins from human plasma by different fractionation methods. Scand. J. Macmatol. *1*: 257-271.

64. ONOUE, K., KISHIMOTO, T. and YAMAMURA, Y., 1967. Papain fragmentation of the subunits of human macroglobulin. J. Immunol. *98*: 303-313.

65. PENNELL, R.B., 1960. Fractionation and isolation of purified components by precipitation methods. In: *The Plasma Proteins*, Vol. 1, pp. 9-42 (ed. Putnam, F.W.) Academic Press, New York and London.

66. PETERSON, E.A. and SOBER, H.A., 1956. Chromatography of proteins. I. Cellulose ion-exchange adsorbents. J. Am. Chem. Soc. *78*: 751-755.

67. POLSON, A., POTGIETER, G.M., LARGIER, J.F., MEARS, G.E.F. and JOUBERT, F.J., 1964. The fractionation of protein mixtures by linear polymers of high molecular weight. Biochim. Biophys. Acta *82*: 463-475.

68. POLSON, A. and RUIZ-BRAVO, C., 1972. Fractionation of plasma with polyethylene glycol. Vox. Sang. *23*: 107-118.

69. PORATH, J. and FLODIN, P., 1959. Gel filtration: A method for desalting and group separation. Nature *183*: 1657-1659.

70. PORATH, J., 1960. Gel filtration of proteins, peptides and amino acids. Biochim. Biophys. Acta *39*: 193-207.

71. PORATH, J. and BENNICH, H., 1962. Recycling chromatography. Arch. Biochem. Biophys., Suppl. *1*: 152-156.

72. PUTNAM, F.W., 1959. Abnormal human serum globulins. I. The nonidentify of macroglobulins. Arch. Biochem. Biophys. *79*: 67-85.

73. PUTNAM, F.W., 1960. Abnormal serum globulins. In: *The Plasma Proteins*, Vol. 2, pp. 345-406 (ed. Putnam, F.W.) Academic Press, New York and London.

74. PUTNAM, F.W., 1965. Structure and function of the plasma proteins. In: *The Proteins*, Vol. 3, pp. 153-267 (ed. Neurath, H.) Academic Press, New York and London.

75. REISNER, C.A. and FRANKLIN, E.C., 1961. Studies of mercaptoethanol-dissociated normal human 19 S γ-globulin and pathologic macroglobulins from patients with macroglobulinemia. J. Immunol. *87*: 654-664.

76. ROWE, D.S., 1963. Human γ-globulin as antigen and antibody. Biochemm J. *88*: 2P.

77. ROWE, D.S. and FAHEY, J.L., 1965. A new class of human immunoglobulins. II. Normal serum IgD. J. Exp. Med. *121*: 185-199.

78. SANDOR, G., 1959. Nature des globulines isoélectriquement peu solubles du sérum. Isolement d'une β_1-globuline distincte de la sidérophiline et ses relations au plasminogène. C.R. Acad. Sci. (Paris) *248*: 3361-3362.

79. SCHULTZE, H.E., HAUPT, H., HEIDE, K., MOSCHLIN, G., SCHMIDTBERGER, R. and SCHWICK, G., 1962. Untersuchungen Über gamma-makroglobuline des menschlichen serum. Z. Naturforsch. *17b*: 313-322.

80. SCHULTZE, H.E. and HEREMANS, J.F., 1966. Survey of the plasma proteins. In: *Molecular Biology of Human Proteins*, Vol. 1, pp. 173-235. Elsevier Publishing Co., Amsterdam-London-New York.

81. SCHULTZE, H.E. and HEREMANS, J.F., 1966. The principal methods for the fractionation of plasma proteins. In: *Molecular Biology of Human Proteins*, Vol. 1, pp. 236-317. Elsevier Publishing Co., Amsterdam-London-New York.

82. SHIMIZU, A., PUTMAN, F.W., PAUL, C., CLAMP, J.R. and JOHNSON, I., 1971. Structure and role of the five glycopeptides of human IgM. Nature New Biol. *231*: 73.

83. SOBER, H.A., GUTTER, F.J., WYCKOFF, M.M. and PETERSON, E.A., 1956. Chromatography of proteins. II. Fractionation of serum proteins on anion-exchange cellulose. J. Am. Chem. Soc. *78*: 756-763.

84. SOBER, H.A. and PETERSON, E.A., 1958. Protein chromatography on ion-exchange cellulose. Fed. Proc. *17*: 1116-1125.

85. SOBER, H.A., HARTLEY, R.W. and CARROLL, W.R., 1965. Fractionation of proteins. In: *The Proteins*, Vol. 3, pp. 2-91 (ed. Neurath, H.) Academic Press, New York and London.

86. SQUIRE, D.G., 1964. A relationship between the molecular weights of macromolecules and their elution volumes based on a model for Sephadex gel filtration. Arch. Biochem. Biophys. *107*: 471-478.

86B. SUMNER, J.B., 1919. J. Biol. Chem. *37*: 137. (Cited by AGRAWAL, B.B.L. and GOLDSTEIN, I.J., 1967. Physical and chemical characterization of concanavalin A, the hemagglutinin from jack bean (*Canavalia ensiformis*). Biochim. Biophys. Acta *133*: 376-379.)

87. SUZUKI, T. and DEUTSCH, H.F., 1967. Dissociation, reaggregation and subunit structure studies of some human γM-globulins. J. Biol. Chem. *242*: 2725-2738.

88. TISELIUS, A., PORATH, J. and ALBERTSSON, P.-A., 1963. Separation and fractionation of macromolecules and particles. Science *141*: 13-20.

89. TOMBS, M.P., COOKE, K.B., BURSTON, D. and MACLAGEN, N.F., 1961. The chromatography of normal serum proteins. Biochem. J. *80*: 284-292.

90. VAERMAN, J.-P., HEREMANS, J.F. and VAERMAN, C., 1963. Studies of the immune globulins of human serum. I. A method for the simultaneous isolation of the three immune globulins (γss, γ_1M and γ_1A) from individual small serum samples. J. Immunol. *91*: 7-10.

91. WEINSTEIN, Y., GIVOL, D. and STRAUSBACH, P., 1972. The fractionation of immunoglobulins with insolubilized concanavalin A. J. Immunol. *109*: 1402-1404.

92. WICKERHAUSER, M. and HAO, Y.L., 1972. Large scale preparation of macroglobulins. Vox. Sang. *23*: 119-125.

93. WIRTH, K., ULLMAN, U., BRAND, K., HUTH, K. and HESS, B., 1965. Analyse von globulinen mit der gelfiltration. Klin. Wochenschr. *43*: 528-532.

APPENDIX

The first antibodies detected after antigenic stimulation are usually in the IgM class. Subsequently they decline and eventually disappear within several weeks to be replaced by antibodies of the IgG class, which persist for long periods of time. However, some antigens such as polysaccharides, preferentially stimulate IgM production and may cause a continued production of IgM without appreciable IgG antibody formation (4). Cold agglutinins, rheumatoid factor and antibodies against the somatic O antigen of gram negative bacteria are typically but not exclusively IgM antibodies (6).

In comparison with IgG, IgM has a greater agglutinating power which can be explained on the basis of its greaternumber of reactive sites. As well, IgM seems to fix complement much more efficiently than IgG, resulting in a better hemolytic and bactericidal effect (4).

The above cited characteristics of IgM have important clinical applications. For example, recent infections can be identified by demonstrating specific antibodies of the IgM class in the serum specimen. Recent infections are usually diagnosed by demonstrating a rise in the antibody titer. However, if observation of this rise is missed, it becomes impossible to distinguish betweem recent infections and previous immunity. A solution to this problem is offered by a demonstration of specific antibodies in the IgM class, since IgM antibodies do not carry long lasting immunity. Bürgin- Wolff et al. (1) separated Rubella IgM, IgG, and IgA antibodies by gel filtration on agarose and determined the hemagglutination-inhibition activity of

each fraction. They found that distrbution of Rubella antibodies in the three major classes of Ig (IgG, IgA, IgM) was characteristic for a recent Rubella infection. This antibody distribution could be found several weeks after the antibody titer reached its maximum. Recent infection can thus be diagnosed over a longer period of time than by determining a rise in antibodies.

Congenital infections can be detected by determining the IgM level in umbilical cord serum. Since IgM does not cross the placenta, increased IgM levels (more than 10 mg /100ml) must occur as a result of IgM synthesis by the foetus in response to antigenic stimulation. Intrauterine infections with Rubella, Toxoplasma, Cytomegalovirus, and Herpes simplex have been found to cause elevated IgM levels. To determine what the actual infection is, it is essential to first separate IgM from the other Ig and secondly to test this IgM for specific antibodies. The demonstration of specific antibodies in serum (together with the raised IgM level), is not sufficient to diagnose a particular infection, since IgG antibodies from previous infections of the mother can cross the placenta (5).

Since the IgM class contains certain antibodies (e.g. against gram negative bacteria) which are not present or are minimally present in the other Ig classes, IgM enriched gammaglobulins have sometimes a more therapeutic effect than the ordinary used gammaglobulin preparations containing only IgG. In fact, infections in patients with dysgammaglobulinemia and agammaglobulinemia are often precipitated by the lack of specific antibodies (e,g. agains Pseudomonas, Salmonella). IgM enriched preparations are in these cases very useful both as a

therapeutic and as a prophylactic agent. Other possible applications include the therapeutic use of enriched IgM preparations in premature infants with physiological deficiencies in IgM production and in patients under chemotherapy and immunosuppressiva, often associated with a fall in serum IgM (2,3).

The increasing importance of IgM as a potential therapeutic agent and a diagnostic aid should be clear from the above discussion. It is equally clear, however, that isolation of IgM for these purposes by the classical protein purification procedures would be too unweildly an approach to use on a routine basis (1). Alternatively, IgM can be isolated from sera much more efficiently and rapidly by an immunoadsorptio technique. One of the primary purposes behind the experiments described in this thesis was to purify normal human IgM which could then be used (either whole, or after digestion and isolation of specific IgM fragments) to stimulate the production of specific anti- human IgM antibodies. As well, the purified IgM could be used as the ligand on a solid - phase immunoadsorbent designed for the purification of those anti - human IgM antibodies. A population of normal human IgM was desired, in contradistinction to a pathological IgM , which could have been purified much more readily, in order to avoid any atypical reactions or interactions which might have arisen out of the use of a pathological Ig for these purposes. Once sufficient quantities of anti - human IgM antibodies had been purified, as described above, an immunoadsorbent employing those antibodies as ligands could be synthesized. Such an immunoadsorbent might be used for the isolation of IgM class antibodies from human serum for diagnostic purposes and similar techniques could

be used for isolating and purifying IgM from other sources for therapeutic applications.

As a general principle, it should be stated that the use of specific adsorption techniques such as immunoadsorption and affinity chromatography, are likely to supplant classical protein purification procedures as the method of choice for that purpose in the near future. The experiments described in this thesis are representative of the initial steps that must be taken (at this time) in the development of an adsorption purification technique for a specific serum protein. It is envisaged that adsorption purification procedures similar to those described above ultimately will have wider and more significant clinical applications than those few possibilities that have been suggested herein.

BIBLIOGRAPHY TO APPENDIX

1. BURGIN-WOLFF, A., HERNANDEZ, R. and JUST, M., 1971. Separation of Rubella IgM, IgA and IgG antibodies by gel filtration on agarose. Lancet: 1278-1280.

2. HOBDAY, J., 1971. Antibody deficiency disease in children, including normal levels of plasma proteins in children. Ann. Gen. Pract.: 46-50.

3. MARDER, E., 1971. Immunoglobulin and hyperimmuno-globulin therapy. Ann. Gen. Pract.: 29-35.

4. PIKE, R., 1967. Antibody heterogeneity and serological reactions. Bacteriol.Rev. 31: 157-174.

5. SEVER, J., 1969. Immunoglobulin determination for the detection of perinatal infections. J. Pediat. 75:1111-1115.

6. WALDMANN, T., 1969. Disorders of immunoglobulin metabolism. New Eng. J. Med. 281: 1170-1177.